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ANALYSIS OF GnRH RECEPTOR GENE EXPRESSION IN LINES OF SWINE WITH DIVERGENT OVULATION RATES

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ANALYSIS OF GnRH RECEPTOR GENE EXPRESSION IN LINES OF SWINE WITH DIVERGENT OVULATION RATES

by

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CHAPTER I

INTRODUCTION

Litter size is one of the most important factors in swine production, averaging 9 piglets per sow in the U.S. (USDA-National Agricultural Statistical Services, 2007). Increasing litter size by 1 piglet per sow could have a dramatic economic impact on the pork industry. For example, given a live basis price of $0.35/lb and a 250 lb market weight, a producer with 10,000 sows could increase income by $875,000 per farrowing. Although many factors influence prolificacy, including ovulation rate, embryonic survival and uterine capacity, ovulation rate is one of the major determinants of litter size (Christenson et al., 1993). Similar to other reproductive traits, ovulation rate is regulated by gonadotropin-releasing hormone (GnRH) binding to its specific receptor on the plasma membrane of gonadotrope cells located in the anterior pituitary gland.

Gonadotropin-releasing hormone is released in a pulsatile manner from hypothalamic neurons and binds to its cognate receptor on the surface of gonadotrope cells within the anterior pituitary gland. This causes the synthesis and secretion of the gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH; Clayton and Catt, 1981; Clarke et al., 1983). The gonadotropins, in turn, act at the level of the gonads to promote gamete maturation, ovulation and steroid hormone production. The steroid hormones may act in a negative or positive feedback manner to control the production of GnRH and the gonadotropins at the level of the hypothalamus and anterior pituitary gland, respectively (Nakai et al., 1978; Conn and Crowley, 1994; McNeilly et

al., 2003). Focusing on the mechanisms within gonadotrope cells, binding of GnRH to its cognate G-protein coupled receptor, stimulates the up-regulation of at least 4 gonadotropic genes; the common glycoprotein hormone α-subunit, the unique β-subunits specific to both LH and FSH (Hamernik and Nett, 1988; Gharib et al., 1990), as well as its own receptor (Sealfon and Millar, 1995). Thus, the interaction between GnRH and its receptor represents a central point for regulation of reproductive function.

In addition to the physiological relevance of the interaction between GnRH and its receptor, the porcine GnRH receptor (GnRHR) gene is located on chromosome 8 and is in close proximity to a quantitative trait locus (QTL) for ovulation rate (Rohrer et al., 1999). Thus, the porcine GnRHR gene represents both a physiological and positional candidate for genes influencing ovulation rate. To study the transcriptional regulation of the porcine GnRHR gene and its possible effects on ovulation rate, our laboratory has utilized 3 lines of swine with divergent ovulation rates; the Control white-crossbred, Index, and Chinese Meishan. The Index line, developed at the University of Nebraska-Lincoln was selected for over 14 generations based on an index of ovulation rate and embryonic survival. These females ovulate approximately 7.4 more oocytes than females from the Control line (Johnson et al., 1999). The Chinese Meishan is a highly prolific breed, producing 4 to 5 more piglets per litter compared to occidental breeds, largely due to ovulation rate (Christenson et al., 1993; White et al., 1993).

To examine the relationship between the molecular mechanisms underlying ovulation rate and transcriptional regulation of the porcine GnRHR gene, our laboratory has isolated the GnRHR gene promoter from the Control, Index and Meishan lines

(McDonald, 2005). Previous work in our laboratory has indicated divergent luciferase activity among reporter vectors containing 5000 bp of 5' flanking region for the porcine GnRHR gene from all three genetic lines transiently transfected into gonadotrope-derived αT3-1 cells (Fig 1.1; McDonald, 2005). This suggests differential mechanisms involved in the transcriptional regulation of the GnRHR gene among pig strains.

Additionally, our laboratory identified a single base alteration between the Meishan and homologous Control/Index promoters located at -1235 bp of 5' flanking region. This single bp substitution allows for the binding of the p65 and p52 subunits of NF-κB and an Sp1-like protein in the Meishan promoter, events that are absent in the Control/Index promoters (McDonald, 2005). Transient transfections with vectors containing block replacement mutations of these binding sites, either singly or in combination, decreased luciferase activity by approximately 25%. Despite a significant reduction in promoter activity, however, luciferase values were not reduced to levels exhibited by reporter constructs containing the full-length Control promoter. This suggests that other unidentified element(s) within the Meishan proximal promoter contribute to its enhanced activity. Therefore, we need to identify additional elements and transcription factors that contribute to the line-specific activity of the porcine GnRHR gene.

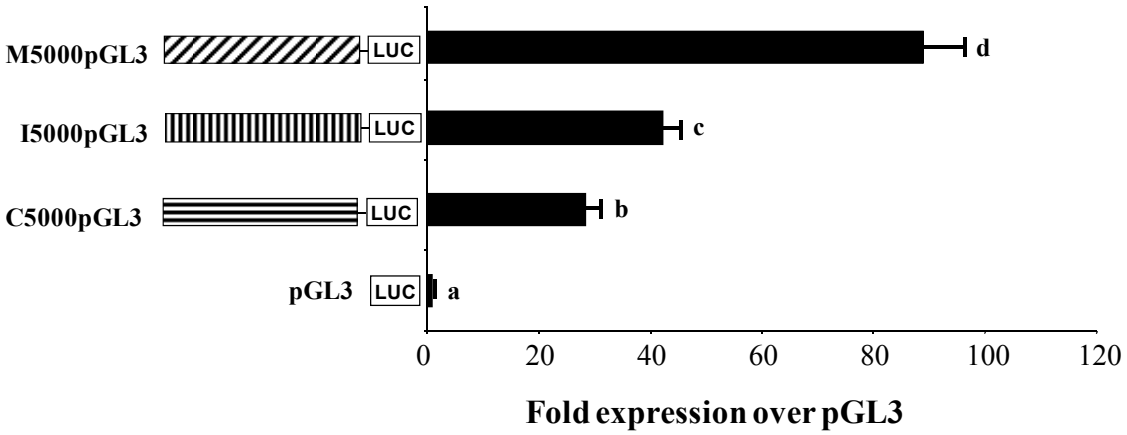


Figure 1.1. Differential GnRHR gene promoter activity among swine lines with divergent ovulation rates. Luciferase reporter constructs containing 5000 bp of proximal promoter for the Index (I5000pGL3) and Meishan (M5000pGL3) GnRHR genes display 2- and 4-fold increased activity compared to those containing the Control (C5000pGL3) promoter. Bars with alternate superscripts differ ($P < 0.05$). From McDonald (2005).

CHAPTER II

LITERATURE REVIEW

Gonadotropin-Releasing Hormone

Role in Reproductive Axis

The decapeptide, gonadotropin-releasing hormone (GnRH), was first isolated from mammalian hypothalamic tissue (Amoss et al., 1971; Matsuo et al., 1971; Schally et al., 1971) and determined to be essential to reproductive function. It is released in a pulsatile manner from the hypothalamus into the hypothalamo-hypophyseal portal vascular system and travels to the anterior pituitary gland (Figure 2.1; Carmel et al., 1976; Levine et al., 1982). Here, GnRH binds to specific G protein-coupled receptors (GPCRs) located on the plasma membrane of gonadotrope cells within the anterior pituitary gland. The GnRH receptor (GnRHR) is a member of the GPCR family, characterized by an extracellular N-terminus and intracellular C-terminus linked by seven transmembrane helices and connected by three extracellular and intracellular loops. However, the GnRHR is a unique member of the GPCR family because it lacks a C-terminal tail (Tsutsumi et al., 1992; Probst et al., 1992; Davidson et al., 1994; Wess, 1997). Gonadotropin-releasing hormone binding to its receptor stimulates the up-regulation of at least 4 gonadotropic genes: the common α-subunit; the unique β-subunits specific to the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone

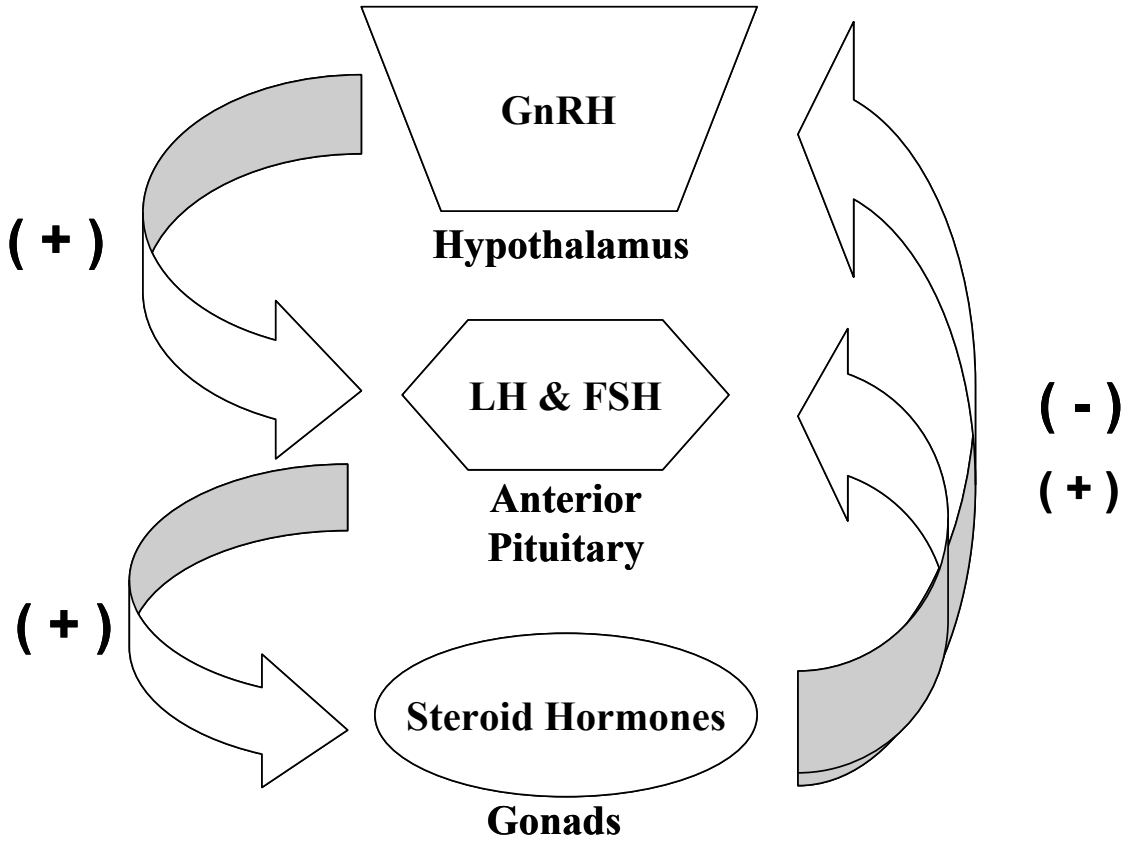


Figure 2.1. Schematic of the reproductive axis. Gonadotropin-releasing hormone (GnRH) is produced in the hypothalamus and acts on gonadotropes within the anterior pituitary gland. This leads to the production of the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH). These hormones, in turn, have an effect on the gonads, causing release of steroid hormones. The steroid hormones then act in a negative or positive feedback loop to control the production of GnRH and the gonadotropins.

(FSH; Hamernik and Nett, 1988; Gharib et al., 1990); and its own receptor (Sealfon and Millar, 1995).

The binding of GnRH to its receptor causes the synthesis and secretion of the gonadotropins, LH and FSH (Figure 2.1; Clayton and Catt, 1981; Clarke et al., 1983). Luteinizing hormone induces ovulation in the female (Velardo, 1960) and testosterone production in the male (Veyssiere et al., 1977), whereas FSH is involved in follicular recruitment and maturation in the female (Velardo, 1960) and spermatogenesis in the male (Weissenberg et al., 1982). Next, the gonadotropins act on the gonads to cause the production of steroid hormones such as androgens, progesterone (P_4) and estradiol-17β (E_2). The steroid hormones can provide negative or positive feedback to control production of both GnRH at the level of the hypothalamus and gonadotropins from the anterior pituitary gland (Figure 2.1; Nakai et al., 1978; Conn and Crowley, 1994; McNeilly et al., 2003).

Production and Secretion

Gonadotropin-releasing hormone is released in a pulsatile manner from hypothalamic neurons and binds to specific receptors on the plasma membrane of gonadotrope cells within the anterior pituitary gland. This specific release of GnRH is a prerequisite for fertility during the female reproductive cycle (Moenter et al., 1991). Gonadotropin-releasing hormone is released in different frequencies throughout the follicular and luteal phases of the estrous cycle. Studies in the ewe have indicated that during the start of the follicular phase, E_2 levels rise causing GnRH pulse frequency to

increase and pulse amplitude to decrease (Clarke et al., 1987; Moenter et al., 1991). During the luteal phase of the estrous cycle, GnRH release in the ewe occurs in high amplitude, low frequency pulses, whereas the frequency of GnRH pulses increased at the end of the luteal phase as P_4 levels decreased (Clarke et al., 1987; Moenter et al., 1991; Clarke, 1995). Administration of GnRH in a pulsatile manner to lactating sows increased LH, stimulating follicular development and inducing sows into estrus (Britt et al., 1985). Bracken and associates (2007) administered GnRH in a pulsatile manner 4 days prior to weaning, for 2 consecutive days, causing a wave of follicular growth that was not sustained post-weaning. Knox and associates (2003) analyzed gilts with high or low ovulation rates and measured the levels of gonadotropins and steroid hormones during their estrous cycle. Swine with elevated ovulation rates had higher FSH levels during the ovulatory period as well as the mid- and late-luteal phases. During the ovulatory period, the gilts with an increased ovulation rate had greater LH levels. Thus, increased synthesis and secretion of GnRH caused an increase in FSH and LH, resulting in amplified ovulation rates during various stages of the estrous cycle (Knox et al., 2003).

Effects of puberty, aging, season/photoperiod, nutrition and stress may cause changes in GnRH synthesis and secretion. During postnatal development in rats (Sisk et al., 2001) and monkeys (Plant and Shahab, 2002), there is a steady increase in the hypothalamic levels of GnRH content and release. Post-pubertal hamsters have increased levels of GnRH mRNA in GnRH neurons within the brain compared to pre-pubertal hamsters, possibly due to an alteration of GnRH neuronal activity or central mechanisms controlling GnRH secretion (Parfitt et al., 1999). Gonadotropin-releasing hormone

secretion can impact the selection of follicles in post-pubertal gilts. Therefore, GnRH secretion may be important in regulating a pulsatile release of LH, critical for follicular growth in post-pubertal gilts (Knox, 2005).

Gonadotropin-releasing hormone secretion is also affected by increasing age, resulting in reduced preovulatory GnRH/LH surges and attenuation of pulsatile LH secretion. Wise et al. (2002) utilized middle-aged rats to understand the interactions between the ovary and brain during menopause. Subtle changes in GnRH secretion in middle-aged rats decreased E_2. Eventually, the inability of GnRH to travel to the anterior pituitary disabled the LH surge, leading to irregular estrous cycles and ultimately ending their reproductive cycles. Therefore, menopausal women are unable to coordinate E_2 with the regular preovulatory GnRH surges, resulting in irregular estrous cycles and eventually acyclicity (Wise et al., 2002). An additional study suggested that a loss of ovarian cyclicity causes an absence of E_2 negative feedback on GnRH neurons within the brain in aging female mice (Thanky et al., 2003).

Seasonal breeders such as sheep, horses and hamsters utilize photoperiod and other cues to regulate their circannual pattern of reproduction (Thiery et al., 2002). Melatonin plays an important role in the regulation of seasonal reproductive behavior, but responsiveness of the hypothalamus to melatonin dictates the difference between long and short day breeders (Gerlach and Aurich, 2000). In long day breeders, (horse and hamster) increased levels of melatonin secretion are associated with decreased GnRH secretion. However, increased melatonin levels are associated with an increase in GnRH in short day breeders (sheep; Grosse et al., 1993). Reproduction is repressed by increased

melatonin in long day breeders, whereas it is stimulated by elevated melatonin in short day breeders (Grosse et al., 2003).

Nutritional status can also have an effect on GnRH production and secretion. For instance, plasma levels of LH are reduced in sheep during food deprivation as a result of inhibitory influences on secretion of GnRH (Henry et al., 2001). Another study suggested that food restricted, male prairie voles had increased numbers of GnRH neurons, as detected by immunohistochemistry, probably due to reduced secretion of GnRH (Kriegsfeld et al., 2001). Restricting food to pre-pubertal bulls delayed puberty, reducing the pituitary response to GnRH and hindering testicular steroidogenesis (Brito et al., 2007). However, supplemental feeding did not stimulate puberty (Brito et al., 2007).

Finally, stress can have detrimental effects on the reproductive axis. Administration of a bacterial endotoxin, which induces immune/inflammatory stress, to ovariectomized ewes reduced pulsatile GnRH/LH secretion (Debus et al., 2002). Lapot and associates (2007) indicated that short and prolonged footshock stimulation to sheep activated both the GnRH and GnRHR genes in the preoptic area of the hypothalamus and the GnRHR gene in the anterior pituitary of anestrous ewes. However, there was in increase in LH secretion in animals that were stressed for a short period of time. Thus, short amounts of stress can have a positive effect on reproduction (Lapot et al., 2007).

Structure of Gonadotropin-Releasing Hormone Isoforms

Gonadotropin-Releasing Hormone. Mammalian GnRH is a decapeptide consisting of the following amino acids: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-

NH_2 (Figure 2.2; Schally et al., 1971; Matsuo et al., 1971; Baba et al., 1971). Gonadotropin-releasing hormone has 23 known isoforms from vertebrate and protochordate species and has been conserved for more than 500 million years of evolution (Millar, 2005). The NH_2-terminal sequence has been highly conserved, suggesting that is critical for both receptor binding and activation, whereas the COOH-terminus is no longer present in the first isoform of GnRH (Millar, 2005). In order to enhance binding affinity for its specific receptor, the mammalian GnRH peptide is folded to a specific tertiary conformation (Millar et al., 2004; Millar, 2005), whereas the non-mammalian GnRH peptide maintains a more extended structure (Guarnieri and Weinstein, 1996; Maliekal et al., 1997). Following subsequent isolation of GnRH isoforms, the original form of GnRH has been termed GnRH-I.

Gonadotropin-Releasing Hormone-II. Another form of GnRH, first identified from chicken brain, is ubiquitous in vertebrates from primitive bony fish to man (GnRH-II; Millar et al., 1987; Millar and King, 1988; Sherwood et al., 1993; White et al., 1994; Lescheid et al., 1997). The second form of GnRH is a decapeptide consisting of the following amino acids: pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH_2. This structure differs from GnRH-I at the fifth, seventh and eighth amino acids (Figure 2.2; Chen et al., 2001). The GnRH-II gene has been isolated in cartilaginous and bony fish (Powell et al., 1986; Lovejoy et al., 1992; Kim et al., 1995; Powell et al., 1996), amphibians (King and Millar, 1986; Licht et al. 1994; Pinelli et al., 1997), reptiles (Powell et al., 1985; Sherwood and Whittier, 1988), birds (Miyamoto et al., 1984; Van-Gils et al., 1993),

GnRH Isoforms	Amino Acid Sequence
GnRH-I	pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2
GnRH-II	pGlu-His-Trp-Ser-**His**-Gly-**Trp**-**Tyr**-Pro-Gly-NH_2
GnRH-III	pGlu-His-Trp-Ser-**His**-**Asp**-**Trp**-**Lys**-Pro-Gly-NH_2

Figure 2.2. Comparison of the 3 isoforms of GnRH. Amino acids in bold are different from the originally identified form, GnRH-I.

monkeys (Lescheid et al., 1997; Urbanski et al., 1999), humans (Chen et al., 1998; White et al., 1998) and pigs (Millar, 2003). Although mice do not apparently make the GnRH-II ligand (Millar, 2003), previous studies identified GnRH-II immunopositive cells in various locations of the mouse and rat brain (Chen et al., 1998; Gestrin et al., 1999). Gonadotropin-releasing hormone-II has been identified in many tissues including the brain, pituitary gland (Chen et al., 2001), kidney, bone marrow (White et al., 1998), immune system (Chen et al., 2002a), and reproductive tissues such as the ovary (Choi et al., 2001), breast (Chen et al., 2002b), uterine endometrium (Cheon et al., 2001) and prostate (White et al., 1998). The GnRH-II ligand contains a preconformed β-II turn, making it more stable than GnRH-I. It may also differ from GnRH-I in its pharmacological bioavailability because GnRH-II is believed to be more susceptible to peptidase degradation (Pawson et al., 2003).

Although the function of GnRH-II is still unknown, unlike GnRH-I, it is likely involved in more biological systems than the reproductive system because of its ubiquitous expression. Kauffman and associates (2006) suggested that food restricted musk shrews expressed fewer reproductive behaviors which was correlated with reduced levels of GnRH-II in various brain nuclei. Barnett and associates (2006) analyzed the reproductive behavior of female marmosets in the presence of a male and concluded that GnRH-II and its receptor play a role in stimulating female reproductive behavior. Additional studies also proposed that GnRH-II may be important for hormone regulation during pregnancy (Siler-Khodr and Grayson, 2001) and maintaining normal ovarian function (Siler-Khodr et al., 2003).

Gonadotropin-Releasing Hormone-III. Additional isoforms of GnRH have been cloned from lamprey; lamprey GnRH-I (l-GnRH-I) and lamprey GnRH-III (l-GnRH-III). Lamprey-GnRH-III (l-GnRH-III) is also a decapeptide with the following amino acid sequence: pGlu-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH_2 (Figure 2.2; Mezo et al., 2007). With 60% homology to GnRH-I, l-GnRH-III contains different amino acids in positions five to eight (Sower et al., 1993). This natural isoform of GnRH isolated from sea lamprey is considered to be a weak agonist of GnRH-I within the pituitary. Lamprey-gonadotropin-releasing hormone-III is not a natural ligand in humans but inhibits the growth of human cancer cells (Heredi-Szabo et al., 2006).

Gonadotropin-Releasing Hormone Receptor

Cloning and Isolation of Gene

Coding Sequence. The GnRHR was first isolated from gonadotrope-derived αT3-1 cells of mouse origin (Tsutsumi et al., 1992). The αT3-1 cell line endogenously produces GnRHRs; therefore, it represents a good cell line for in vitro studies of the GnRHR (Windle et al., 1990). Following isolation of the mouse GnRHR, it was identified in other mammalian species including the cow (Kakar et al., 1993), human (Chi et al., 1993; Kakar et al., 1992), pig (Weenser and Matteri, 1994), sheep (Illing et al., 1993; Brooks et al., 1993), marmoset (Byrne et al., 1999), rat (Eidne et al., 1992; Kaiser et al., 1992) and guinea pig (Fujii et al., 2004). Some differences are present in the GnRHR cDNA sequence among these species but their amino acid sequence has 85%

homology to the mouse GnRHR. One of the differences among these species is the addition of one amino acid in the cow, human, pig and sheep in comparison to the rat and mouse (Cheng and Leung, 2000).

The GnRHR is highly conserved among mammalian species (Kakar et al., 2002). For example, the rat and mouse GnRHR cDNAs are very similar (Millar, 2005; Schneider et al., 2006). The human GnRHR contains 328 amino acids and is 90% identical to the mouse GnRHR (Chi et al., 1993). The bovine GnRHR also contains 328 amino acids, exhibiting 91 and 86% identity to the human and mouse/rat receptors, respectively (Kakar et al., 1993). The porcine, bovine and ovine GnRHRs are comparable in length to the human, with all containing an additional amino acid at position 191 in the second extracellular loop that is not present in rodents (Schneider et al., 2006; Millar, 2005; Weenser and Matteri, 1994). The porcine GnRHR is 88% identical to the bovine and ovine, 87% similar to the human, and has 81% homology to the rat. In addition, the marmoset GnRHR has an 85 and 95% homology to the rat and human GnRHR, respectively (Schneider et al., 2006).

5' Flanking Sequence. The 5' flanking region of the GnRHR gene has been isolated in many different mammalian species such as mouse (Albarracin et al., 1994), rat (Reinhart et al., 1997; Pincas et al., 1998), human (Fan et al., 1995; Kakar, 1997), sheep (Campion et al., 1996), and pig (Jiang et al., 2001; Cederberg et al., unpublished data). Isolation of the GnRHR gene promoter from various species has led to examination of the transcriptional regulation of this gene. The promoters among each of species contain

some homology. For instance, the rat and mouse promoters share 80% homology, whereas the rat promoter shares 55% homology with the human promoter. In addition to sharing homology with the human promoter, the rat also shares 63% homology with the sheep promoter (Pincas et al., 1998). Within 500 bp of proximal promoters from the mouse, rat, human, sheep and pig there are several highly homologous regions (Pincas et al., 1998).

The mouse GnRHR 5' flanking sequence was the first to be analyzed. The transcriptional start site is located at -62 bp, not in proximity to a consensus TATA box (Albarracin et al., 1994). The transcriptional start site for the rat GnRHR proximal promoter is located 103 bp upstream from the start codon, with a putative TATA box situated 23 bp upstream from the transcriptional start site (Reinhart et al., 1997). However, the human and sheep GnRHR proximal promoters are more complex compared to the rat and mouse. For example, they contain multiple transcriptional start and catabolite activator protein (CAP) sites (Campion et al., 1996; Ngan et al., 2000). The sheep GnRHR promoter is similar to the mouse; however, it is more homologous to the human promoter sequence (Campion et al., 1996). The human promoter contains 18 transcription initiation sites and several TATA and CAAT boxes located close to one another (Fan et al., 1995; Kakar, 1997) compared to the mouse that only contains one transcriptional start site (Sadie et al., 2003). Lastly, Jiang et al. (2001) isolated approximately 1200 bp of the porcine GnRHR proximal promoter. The porcine GnRHR 5' flanking sequence contains 7 putative CAAT boxes and 3 potential TATA boxes in close proximity to each other.

Structure of Gonadotropin-Releasing Hormone Receptor Isoforms

Gonadotropin-Releasing Hormone Receptor. The GnRHR is a member of the GPCR family which is characterized by an extracellular N-terminus and an intracellular C-terminus. These termini are linked by 7 transmembrane helices which are connected by 3 intracellular and extracellular loops (Reinhart et al., 1992; Wess, 1997; Ji et al., 1998). These intracellular and extracellular loops are important for ligand binding and signal transduction, respectively (Counis et al., 2005). However, the GnRHR also has a short third intracellular loop and lacks an intracellular C-terminal tail (Figure 2.3; Tsutsumi et al., 1992; Probst et al., 1992; Davidson et al., 1994; Wess, 1997). The absence of a C-terminal tail is unique to the mammalian GnRHR because it is present in all other G-protein coupled and non-mammalian GnRHRs. Addition of a C-terminal tail to the rat GnRHR resulted in increased internalization rates. Thus, the lack of an intracellular C-terminal tail in the mammalian GnRHR may help maintain many active receptors on the surface of gonadotrope cells following a GnRH surge (Heding et al., 1998).

Gonadotropin-Releasing Hormone Receptor-II. An additional member of the GnRHR family of GPCRs is GnRHR-II. Unlike the first form of GnRHR, GnRHR-II contains a C-terminal tail, critical for receptor internalization (Figure 2.3; Faurholm et al., 2007; Ronacher et al., 2004). The cDNA and genomic sequences for the human GnRHR-II have been isolated (Faurholm et al., 2001; Morgan et al., 2003). Additionally, the pig (Neill et al., 2004), marmoset (Millar et al., 2001), African green monkey and rhesus

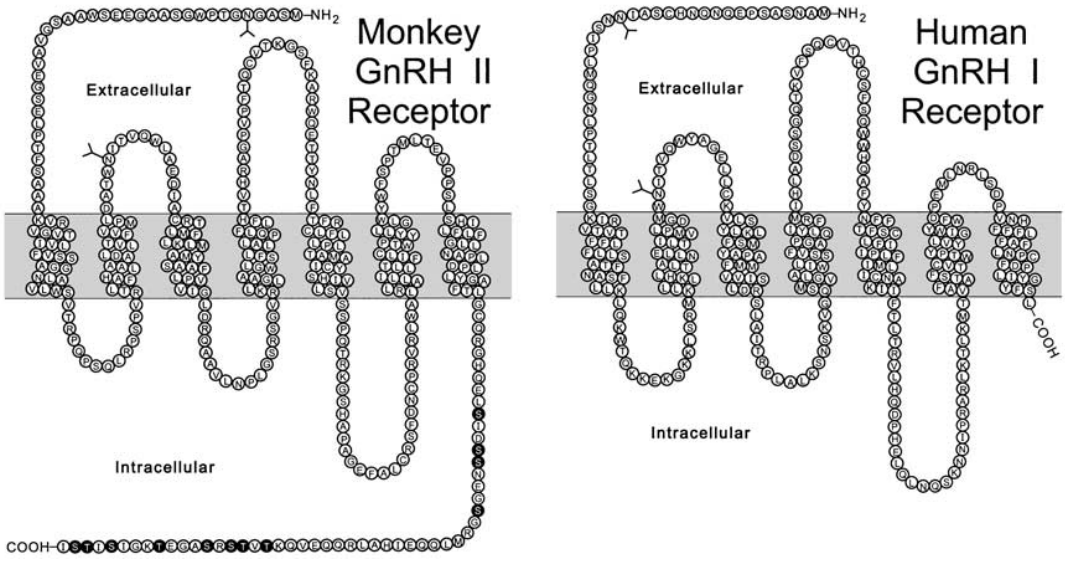


Figure 2.3. Structural diagram of the receptors specific to GnRH-I and -II. Gonadotropin-releasing hormone receptor-II contains an intracellular C-terminal tail that is critical for receptor internalization. However, GnRHR-I lacks an intracellular, C-terminal tail. From Neill (2002).

monkey (Neill et al., 2001) GnRHR-II cDNA sequences have been identified. Only the genes encoding GnRHR-II have been sequenced in the sheep (Gault et al., 2004), bovine (Morgan et al., 2006), and chimpanzee (Morgan and Millar, 2004) and coding regions have been predicted. Sheep and bovine GnRHR-II have an 80 and 67% amino acid identity, respectively, whereas both sheep and bovine have a 41% amino acid homology to human GnRHR-II. Most primates have a 90-93% amino acid homology amongst each other for GnRHR-II (Faurholm et al., 2007). In addition, the pig and old world monkey GnRHR-II proteins have a 91% amino acid homology (Neill et al., 2004).

The marmoset, African green monkey, rhesus monkey, and pig gene sequences produce functional GnRHR-IIs. The marmoset GnRHR-II gene contains 3 exons and 2 introns with a similar structure to the mammalian GnRHR-I gene (Faurholm et al., 2007). Neill et al. (2004) isolated and sequenced cDNA encoding a protein for the 7 transmembrane porcine GnRHR-II. Additionally, they identified a 5 transmembrane receptor formed by alternative splicing in exon 1. However, the bovine, sheep, human, and chimpanzee GnRHR-II genes encode a non-functional protein (Faurholm et al., 2007). The human GnRHR-II contains a premature stop codon (UAA) that is located in frame within exon 2, suggesting the receptor is non-functional (Morgan et al., 2003). The premature codon identified within the human GnRHR-II gene is also conserved in the chimpanzee (Morgan et al., 2003). Additionally, the human and chimpanzee genes have the same stop codon in exon 2 and the same frame shift in exon 1 (Faurholm et al., 2007). The bovine GnRHR-II gene has a 2 bp deletion within exon 1, resulting in a frame shift. In comparison to the sheep GnRHR-II gene, the bovine has an alteration of 21 codons in

the first exon, including 5 silent changes, 14 amino acid modifications and 2 amino acid deletions (Morgan et al., 2006). The sheep has a major deletion in exon 2 and a premature stop codon in exon 1, whereas the bovine contains a premature stop codon in exon 2 (Morgan et al., 2003).

Other Isoforms. Moncaut and associates (2005) identified 5 GnRHRs in teleost fish. Gonadotropin-releasing hormone and GnRHRs are important in cellular functions other than the classical role within the anterior pituitary gland (Moncaut et al., 2005). The lamprey GnRH receptor (l-GnRHR) is highly unique because it has the longest intracellular C-terminal tail (120 amino acids) of any previously identified GnRHR (Silver et al., 2005). This unusual C-terminal tail may be required for efficient ligand binding, stimulation of another unknown signaling pathway and/or structural stability. Activation of this receptor with either l-GnRH-I or l-GnRH-III promoted cAMP production in a dose-dependent manner. Additionally, the l-GnRHR displayed rapid ligand-dependent internalization compared to the truncated tailless form of l-GnRH-III (Silver et al., 2005; Silver and Sower, 2006). The l-GnRHR has similar characteristics of both GnRHR-I and -II, therefore it has maintained ancestral characteristics of the vertebrate GnRHR family. However, lampreys have become a unique model system for analyzing and understanding the evolution of the neuroendocrine regulation of reproduction (Sower, 2003; Silver and Sower 2006).

Tissue Expression

Anterior Pituitary. The GnRHR gene is expressed in gonadotrope cells of the mammalian anterior pituitary gland and plays an important role in the reproductive axis. The number of GnRHRs within the anterior pituitary gland is regulated during fetal development (Granger et al., 2004), sexual maturation (Zapatero-Caballero et al., 2003) and the reproductive cycle (Bauer-Dantoin et al., 1993). During fetal development, GnRHR gene expression is regulated by changes in levels of E_2 and P_4 as well as an increase in GnRH secretion (Granger et al., 2004). Zapatero-Caballero and associates (2003) determined that the activation of genes for GnRHR and gonadotropic subunits within the anterior pituitary gland depended on GnRH secretion, contributing to sexual development in male rats. Also, the number of GnRHRs on gonadotrope cells varies during the rat estrous cycle. More receptors were present during the follicular phase of the estrous cycle than the luteal phase, suggesting a positive effect of E_2 and a negative effect of P_4 on GnRHR gene expression (Bauer-Dantoin et al., 1993).

Gonadotropin-releasing hormone secretion by the hypothalamus may control the number of GnRHRs within the anterior pituitary by increasing mRNA levels, protein amounts or a combination of the two (Clayton and Catt, 1981). Administration of a GnRH antagonist *in vivo* induced a reduction in pituitary GnRHR mRNA levels (Pinski et al., 1996). Furthermore, treatment with increasing concentrations of a GnRH agonist elevated GnRHR mRNA levels in a dose-dependent manner, eventually resulting in decreased expression of the GnRHR gene due to desensitization (Lerrant et al., 1995). Alternatively, administration of GnRH agonists to αT3-1 cells at low concentrations

stimulated, whereas high concentrations inhibited translation of GnRHR mRNA without changing GnRHR mRNA levels (Tsutsumi et al., 1995). Thus, the regulation of GnRHR numbers within the anterior pituitary gland may utilize both transcriptional (Tsutsumi et al., 1993) and post-transcriptional mechanisms (Tsutsumi et al., 1995).

Many studies investigating expression of the GnRHR gene have been performed in gonadotrope-derived cell lines (Windle et al., 1990; Thomas et al., 1996). Weiss et al. (2006) indicated that treatment of αT3-1 cells with E_2 or E_2 in combination with P_4 decreased GnRHR mRNA levels. Additionally, administration of the GnRH agonist, triptorelin, in a pulsatile fashion, increased GnRHR mRNA levels. However, continuous treatment with triptorelin eventually resulted in decreased GnRHR mRNA amounts due to desensitization (Weiss et al., 2006). Mason and associates (1994) also indicated that continuous administration of the GnRH agonist, des-Gly^{10}-[D-Ala^{6}] GnRH N-ethylamide, in αT3-1 cells down-regulated GnRHR mRNA levels. Conversely, other studies have demonstrated that there was no alteration in levels of GnRHR mRNA following a continuous treatment with the GnRH agonist, [im-Bzl-D-His^{6}, Pro^{9}-N-ethyl-amide] GnRH, in αT3-1 cells (Tsutsumi et al., 1993; Alarid and Mellon, 1995). Therefore, these contradictory results suggest that GnRHR mRNA levels depend on both the type and concentration of GnRH agonist being administered.

Fewer studies analyzing the expression of the GnRHR gene have been performed in the LβT2 cell line. Similar to the characteristics of normal pituitary cells, LβT2 cells treated with either GnRH or steroid hormones altered GnRHR gene expression (Turgeon et al., 1996). Administration of GnRH in a pulsatile manner to LβT2 cells increased

GnRHR mRNA levels (Turgeon et al., 1996), similar to that of αT3-1 cells (Weiss et al., 2006). Conversely, treatment with E_2 alone did not significantly increase GnRHR mRNA levels. However, in combination with a dexamethasone treatment, GnRHR mRNA levels were increased by E_2, suggesting that E_2 may need to interact with other factors in order to maintain or increase GnRHR mRNA levels (Turgeon et al., 1996).

Hypothalamus. To date, very few studies have been performed to identify the role of GnRHRs within the hypothalamus. Gonadotropin-releasing hormone receptor mRNA has been detected in human neuronal cells (GT1-7 and TE-671). Li et al. (1996) reported that treatment of GT1-7 neurons with human chorionic gonadotropin (hCG) down-regulated expression of the GnRHR gene via a post-transcriptional mechanism. Due to decreased numbers of GnRHRs, LH/hCG may alter the self-stimulatory mechanism of GnRH synthesis and secretion in hypothalamic neurons (Li et al., 1996). Treatment of TE-671 cells with a GnRH agonist caused the phosphorylation of extracellular signal-regulated kinase (ERK1/2) and jun N-terminal kinase (JNK), suggesting that functional GnRHRs are expressed within this cell line. Also, treatment of TE-671 cells with the protein kinase C (PKC) inhibitor, staurosporine, stimulated GnRHR promoter activity. Therefore, the GnRHR gene is down-regulated by the PKC pathway within TE-671 cells (Yeung et al., 2005). Additionally, deletion of two specific regions within the human GnRHR promoter located at -1300/-1018 and -2197/-1900 bp decreased promoter activity within the TE-671 cell line, indicating that there are

important basal regulatory elements within these promoter sequences (Yeung et al., 2005).

In addition to neuronal cell lines, GnRHR mRNA has been detected in native hypothalamic neurons. Wilson and associates (2006) detected GnRHR on pyramidal neurons of the hippocampus, neocortical neurons of the entorhinal cortex and occipitotemporal gyrus of the human brain. The presence of GnRHR in these regions of the human brain suggests that GnRH signaling may play a critical role in the function of hippocampal neurons (Wilson et al., 2006). However, the function of GnRH signaling through receptors within neurons of the brain is still unknown.

Placenta. Gonadotropin-releasing hormone and its receptor are also present in the human placenta and both are structurally and biochemically identical to their hypothalamic and pituitary counterparts, respectively. However, GnRH binds with a lower affinity to receptors in the placenta compared to the anterior pituitary gland (Currie et al., 1981; Escher et al., 1988; Bramley et al., 1992). Gonadotropin-releasing hormone may be associated with hCG secretion within the placenta because hCG secretion has been inhibited in the presence of a GnRH antagonist, suggesting that it may be regulated through a GnRHR-mediated process (Cheng et al., 2001b). Studies indicated that GnRHR expression may be regulated at the transcriptional level in the placenta by the activation of protein kinase A (PKA) and C pathways (Cheng et al., 2000a). This conclusion was made because a GnRH-induced increase in GnRHR expression was attenuated as a result of treatment with PKC, adenylate cyclase or PKA inhibitors.

Likewise, up-regulation of GnRHR mRNA in placental cells occurred following treatment with either a GnRH agonist or forskolin, whereas GnRHR mRNA levels in gonadotrope-derived αT3-1 and ovarian cells decreased following both stimuli. Therefore, regulation of the GnRHR gene is different in the placenta compared to the pituitary (Cheng et al., 2000a) and ovary (Peng et al., 1994; Kang et al., 2000).

Human GnRHR mRNA has been detected in various placental cell lines including: a choriocarcinoma cell line (JEG-3), immortalized extravillous trophoblasts (IEVT) and first trimester cytotrophoblast cells in primary culture (Cheng et al., 2000a; Cheng et al., 2001b). In 1995, Lin and associates isolated GnRHR mRNA from both syncytiotrophoblast and cytotrophoblast layers of the placenta. Consistent with this, both of these layers of the placenta expressed the common glycoprotein α- and specific hCGβ-subunits. This indicated that GnRH regulates hCG secretion by both autocrine and paracrine modes of action (Lin et al., 1995). Using transient transfection and gel mobility shift assays in JEG-3 cells, these investigators identified 4 putative elements located in upstream promoter regions of the human GnRHR gene including: octamer transcription factor-1 (Oct-1), cAMP response element (CRE), GATA, and activating protein-1 (AP-1). However, only the CRE and GATA binding sites were determined to be placental specific (Cheng et al., 2001b).

Ovary. Both GnRH and its receptor have also been identified in the ovary, regulating steroidogenesis (Hsueh and Jones, 1981), oocyte maturation, ovulation (Hillensjo and LeMaire, 1980), follicular atresia/apoptosis (Billig et al., 1994) and gene

expression (Richards, 1994). Investigation of downstream signaling events indicated that binding of GnRH to GnRHR stimulates mitogen-activated protein kinase (MAPK) cascades in ovarian tissues. Additionally, GnRH inhibits FSH-induced, cAMP-dependent responses in ovarian function (Leung and Steele, 1992). Thus, similar to the placenta, GnRH may regulate reproductive mechanisms in the ovary by an autocrine and/or paracrine factor (Cheng et al., 2002a).

In addition, mRNA for GnRH and GnRHR have been isolated from human (Latouche et al., 1989; Peng et al., 1994) and rat granulosa (Billig et al., 1994), immortalized human granulosa-luteal, (SVOG-4o and SVOG-4m; Cheng et al., 2002a), preovulatory rat granulosa (Olofsson et al., 1995) human ovarian (OSE; Kang et al., 2000; Choi et al., 2001) and human luteal cells (Popkin et al., 1983; Bramley et al., 1985). Studies utilized these various ovarian cell lines to analyze GnRHRs within the ovary. Olofsson et al. (1995) indicated that GnRHR mRNA levels are negatively influenced by LH and not affected by FSH. However, GnRH stimulation resulted in increased GnRHR mRNA levels, suggesting an important role for ovarian GnRHR before, during and after ovulation (Olofsson et al., 1995). Peng and associates (1994) demonstrated that GnRH up-regulated GnRHR gene expression, whereas hCG down-regulated GnRHR gene expression. Lastly, Kang and associates (2000) suggested that a growth inhibitory effect regulated via the GnRHR in human OSE cells may cause GnRH to act as an autocrine/paracrine regulator.

In addition to hormonal regulation of GnRHR mRNA levels, basal expression of the GnRHR gene has also been studied in ovarian cell lines. Cheng et al. (2002a)

identified two specific binding sites, C/EBP and GATA, important in regulating basal expression of the GnRHR gene in human granulosa-luteal cells. In addition to their function within ovarian cells, GnRH and GnRHR also play an important role in the ovary during development. For instance, GnRH and GnRHR mRNA levels increased during late fetal development within ovary. Therefore, ovarian GnRH and its receptor may be important in the regulation of gonadal development (Botte et al., 1998).

Testis. In addition to the ovary, GnRH and its receptor have been found in the testis. Previous studies have isolated GnRH-like substances from the interstitial fluid of rat testis (Petersson et al., 1989). Receptors are located in the gonads of adult rats (Hseuh and Jones, 1981) and in rat and mouse testicular germ cells (Bull et al., 2000). Receptor binding studies indicated that a GnRH agonist bound to human testicular tissue, specifically on Leydig cells within the interstitial space of the testis (Clayton et al., 1980; Lefebvre et al., 1980; Sharpe and Fraser, 1980). These receptors in the testis had an identical mRNA sequence to pituitary GnRHRs (Botte et al., 1998). Also, GnRH analogs negatively influenced GnRHR mRNA levels by direct binding to testicular GnRHRs (Botte et al., 1999). The presence of specific GnRHRs in Leydig cells could potentially play a role in the anti-fertility effects of GnRH agonists as well as physiological control of testicular function (Lefebvre et al., 1980). Bahk and associates (1995) suggested that GnRH produced in Sertoli cells could react with GnRHRs in Leydig cells via a paracrine action. To examine the effect of LH, which also has receptors within Leydig cells, on GnRHR gene expression, Botte et al. (1999) administered hCG, an agonist of LH, to

adult male rats. The administration of hCG resulted in decreased GnRHR mRNA levels in the testis. Following testosterone administration, GnRHR mRNA levels increased in the testis, an effect that may be related to negative feedback of testosterone on LH release (Botte et al., 1999). Thus, LH could inhibit GnRHR gene expression or mRNA stability in the testis (Botte et al., 1999). In addition to the role of GnRH and its receptor in testicular function, the GnRHR gene is expressed during early fetal development within the testis. Similarly, GnRH and GnRHR mRNA levels increased within the testis during development. Therefore, testicular GnRH and its receptor may play a potential role in regulation of gonadal development (Botte et al., 1998).

The regulation of GnRHR mRNA levels is different in the testis compared to the anterior pituitary gland. For instance, treatment with a GnRH agonist induced an earlier and more prolonged inhibition of GnRHR mRNA levels within the pituitary (Kaiser et al., 1993) compared to the testis (Botte et al., 1999). Also, GnRH antagonists increased GnRHR mRNA levels in the testis (Botte et al., 1999), whereas they decreased expression of the receptor in the pituitary (Kaiser et al., 1993). Characteristics of testicular GnRHR binding sites are low affinity and high capacity, indicating less specific binding sites in comparison to the classical receptor (Petersson et al., 1989). Thus, GnRHRs within the anterior pituitary may have a different function compared to the GnRHRs within the testis.

Other Tissues. In addition to the anterior pituitary gland, GnRHR has been isolated in various tissues such as: breast (Casan et al., 1998), prostate (Dondi et al.,

1994), and uterine endometrium (Casan et al., 1998; Raga et al., 1998) and myometrium (Chegini et al., 1996). Furthermore, the receptor has been detected in cancer cell lines derived from the pituitary (La Rosa et al., 2000), prostate (Dondi et al., 1994), uterine endometrium (Emons et al., 2000), ovary (Yin et al., 1998; Emons et al., 2000), breast (Sedgley et al., 2006) and placenta (Cheng et al., 2000a; Cheng et al., 2001b). In addition to these tissue types, mRNA for GnRH and its receptor have also been found in rat oocytes (Dekel et al., 1988) as well as developing murine (Raga et al., 1999) and human (Casan et al., 1999) pre-implantation embryos.

Cancer Cell Lines. In addition to normal tissues, various cancer cell types also express GnRHRs. Treatment of cancer cell lines with GnRH agonists and antagonists can inhibit their proliferation, potentially leading to new therapeutic options in cancer therapy (Emons et al., 2000). Gonadotropin-releasing hormone receptor mRNA is present in a breast cancer cell line (MCF7; Sedgley et al., 2006). The human GnRHR is present mostly within the endoplasmic reticulum in both MCF7 and gonadotrope-derived αT4 cells. However, signaling in MCF7 cells allows for the human GnRHR, an intracellular protein, to traffic to the cell surface. Presence of GnRHRs on the cell surface is dependent on both the receptor and cell type. For instance, the receptor type (type I GnRHR without a C-terminal tail, type II GnRHR with a C-terminal tail or type I GnRHR with an added C-terminal tail) varied in expression of GnRHR on the cell surface (Sedgley et al., 2006). It was determined that human GnRHRs had a lower affinity for GnRH compared to mouse and sheep GnRHRs, type II marmoset GnRHRs

and chimeric GnRHRs with added C-terminal tails in MCF7 cells. In addition to the presence or absence of a C-terminal tail, glycosylation within the amino-terminal sequence can affect the number of GnRHRs at the cell surface. Cell type (such as MCF7 or αT4 cells) also affected the number of GnRHRs on the cell surface. Receptor numbers were increased 10-fold in αT4 cells compared to MCF7 cells. However, addition of a C-terminal tail increased receptor binding by 20-fold in MCF cells but only 5-fold in αT4 cells (Sedgley et al., 2006).

Gonadotropin-releasing hormone receptor mRNA has also been found in ovarian cancer cell lines (OVCAR-3 and SKOV-3; Yin et al., 1998; EFO-21, EFO-27; Emons et al., 2000). It has been suggested that approximately 80% of human ovarian cancer cell lines express both GnRH and its receptor (Emons et al., 2000). In addition, GnRHR mRNA has also been detected in a prostate cancer cell line (DU 145; Dondi et al., 1994). Treatment with a GnRH antagonist resulted in increased cell proliferation, suggesting an inhibitory role for GnRH in the regulation of cell growth via an autocrine and/or paracrine mechanism (Dondi et al., 1994; Emons et al., 2000). In addition, Cheng et al. (2000a) isolated GnRHR mRNA from a placental choriocarcinoma cell line (JEG-3). Sequence analysis of the placental GnRHR mRNA indicated that it was 100% identical to its pituitary counterpart. Treatment with a GnRH agonist resulted in an up-regulation of GnRHR mRNA levels in the placental choriocarcinoma cell line compared to down-regulation of GnRHR mRNA levels in pituitary-derived αT3-1 cells. Thus, there may be a different mechanism of regulation in the placenta compared to the pituitary.

Finally, La Rosa and associates (2000) detected GnRHRs in normal pituitary cells and pituitary adenomas. Growth hormone, FSH/LH, adrenocorticotropic hormone, thyroid-stimulating hormone, prolactin and non-hormone secreting (α-subunit/null) cells identified within pituitary adenomas expressed GnRHR mRNA and protein, suggesting that GnRHR may be present in somatotrope, gonadotrope, corticotrope, thyrotrope and lactotrope cells. Therefore, there may be other roles for GnRHRs within the pituitary as well as regulation of gonadotropins. In addition to the GnRHR, normal and adenomatous pituitary cells also express GnRH. Thus, the interaction between GnRH and its receptor may have a role in the regulation of these cell types through a paracrine and/or autocrine mechanism (La Rosa et al., 2000).

Activation of Cell Signaling Pathways Following GnRH Binding

G-protein Coupled Receptors. G-protein coupled receptors activate several cell signal transduction pathways. The stimulus that activates these receptors is a small peptide, the ligand, which binds to the N-terminus (Fredriksson and Schioth, 2005; Ridge and Palczewski, 2007). The characteristic structure of GPCRs includes 7 transmembrane domains that are connected by intracellular loops. It has been suggested that portions of these loops are adequate to bind and activate G-proteins. An example of a GPCR is rhodopsin, a highly organized GPCR important for biosynthesis of photoreceptor cells and optimal activation and signaling involved in light perception (Ridge and Palczewski, 2007). Rhodopsin is made up of 7 transmembrane helices linked together by 3 extracellular and 3 cytoplasmic loops. The N-terminal tail is extracellular and it also

contains a cytoplasmic C-terminal tail (Sakmar, 2002). Metarhodopsin II is the active form of rhodopsin which stimulates changes to the retinal G-protein, transducin (G_t; Ridge et al., 2003). Transducin is responsible for initiating a biochemical cascade of reactions in the process of phototransduction (Ridge and Palczewski, 2007). Another example of a unique GPCR is the GnRHR. Gonadotropin-releasing hormone receptors have an N-terminus situated near 7 α-helical transmembrane domains that are connected by 3 intracellular and 3 extracellular loop domains (Baldwin, 1993; Ballesteros et al., 1998; Miller, 2005). The intracellular loop domains interact with G-proteins and other proteins involved in signal transduction pathways and the extracellular loop domains are involved in conformational changes to assist in receptor activation (Baldwin, 1993; Ballesteros et al., 1998; Millar, 2005).

Most GPCRs, including the non-mammalian GnRHR, contain a C-terminal tail. However, the mammalian GnRHR is an exception, lacking a cytoplasmic tail. In addition, the mammalian GnRHR possesses a relatively short third intracellular loop. Both of these features are important for desensitization of many other GPCRs (Davidson et al, 1994; Cheng and Leung, 2000). Unlike most GPCRS, the mammalian GnRHR does not rapidly desensitize inositol (1,4,5)-triphosphate (IP_3) production and has very slow internalization kinetics (Heding et al., 1998) because the signal promoting internalization is located on the C-terminal tail. Introduction of the C-terminal tail into the rat GnRHR increased internalization rates. This implied that the lack of a C-terminal tail may be important to maintain large numbers of active receptors on the plasma membranes of gonadotropes following the GnRH surge (Heding et al., 1998).

G-proteins. G-proteins are attached to the intracellular surface of cell membranes that contain GPCRs and are activated once the receptor is bound to a ligand. However, inactive G-proteins are heterodimers that consist of α, β, and γ subunits (Koelle, 1997). When GnRH binds to its GPCR, it induces a conformational change in the receptor, allowing the exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the G_α subunit. Subsequently, dissociation of the G_α subunit from the $G_{\beta\gamma}$ dimer occurs, releasing the G_α subunit to activate different signaling cascades within the cell (Koelle, 1997; Millar et al., 2004). The receptor is bound to the specific G-protein subunit leading to activation. Eventually the attached GTP will hydrolyze to GDP and the G_α subunit and $G_{\beta\gamma}$ dimer will re-associate into its inactive heterodimer, starting a new cycle (Koelle, 1997; Millar et al., 2004).

The complex consisting of hormone, receptor, and G-protein is related to the active conformation of GPCRs (De Lean et al., 1980; Samama et al., 1993). Receptor activation is dependent on the formation of a "tertiary complex" in addition to isomerization. For instance, receptors rotate between active and inactive regulatory conformation (Samama et al., 1993). The complex dissociates and the receptor returns to low affinity conformation upon binding of GTP to the G-protein. However, GnRH analogues and intracellular signaling pathways are selected by different GnRHR active conformations (Millar, 2005).

Protein Kinase C. The PKC family of serine/threonine protein kinases is comprised of at least 11 isoenzymes (Nishizuka, 1992; Junoy et al., 2002). These

isoforms play an important role in proliferation, differentiation, gene expression, secretion and activation of signal transduction pathways (Nishizuka, 1992). Isoforms of PKC have been classified into three categories: conventional (α, βI, βII and γ; Nishizuka, 1992; Dutil et al., 1998), novel (δ, ε, η and θ; Gschwendt, 1999), and atypical (ζ and ι/λ; Zhou et al., 1994). Conventional PKCs are required for the activation of (1,2)-diacylglycerol (DAG) and calcium (Nishizuka, 1992; Dutil et al., 1998), novel PKCs do not respond to Ca^{2+} but are sensitive to DAG (Gschwendt, 1999) and atypical PKCs are regulated through phosphoinositides (Zhou et al., 1994). Junoy and associates (2002) studied the down-regulation of specific PKC isoforms following treatment of primary pituitary cell cultures, αT3-1 and LβT2 cells with either a GnRH agonist or a PKC activator (12-O-tetradecanoyl-phorbol-13 acetate; TPA) continuously for 2 to 6 hours. The PKC isoforms α, βII, δ and ε were down-regulated by TPA in LβT2 and primary pituitary cell cultures. However, TPA down-regulated the PKC isoforms α and ε in αT3-1 cells. Treatment with a GnRH agonist down-regulated the PKC isoforms ε and δ in LβT2 cells, whereas only PKC ε was depleted in αT3-1 cells. However, PKC ζ remained resistant in primary pituitary cell cultures, αT3-1 and LβT2 cell lines following treatment with either TPA or a GnRH agonist. Additionally, treatment with proteasome inhibitors (proteasome inhibitor I and II, lactcystin, β-lactone and calpain inhibitor I) prevented TPA and GnRH down-regulation of PKC isoforms in both cell lines. Therefore, the relationship between kinase and proteolytic proteasomal activities that lead to PKC degradation may be part of an important regulatory mechanism involved in a timed and controlled response to different stimuli in gonadotrope cells (Junoy et al., 2002).

The classical pathway activated following binding of GnRH to its receptor is the PKC pathway (Figure 2.4). Previous studies indicated that GnRHR couples to various G-proteins in different cell lines (Reiss et al., 1997; Cheng et al., 2000b; Chamson-Reig et al., 2003). However, to detect which G-proteins coupled to GnRHR *in vivo*, the GnRHR was studied in primary pituitary cell cultures. For example, Stanislaus and associates (1997) detected that the G-protein complex, $G_{q/11\alpha}$ was regulated by GnRH, in primary pituitary cell cultures and GGH_3 cells. Therefore, GnRH binding to its receptor stimulates the G-protein complex, $G_q/_{11\alpha}$, which activates the membrane bound protein, phospholipase Cβ (PLCβ; Cheng et al., 2000b; Chamson-Reig et al., 2003). Active PLCβ hydrolyzes phosphatidylinositol (4,5)-bisphosphate (PIP_2) to generate the second messengers, IP_3 and DAG. Inositol (1,4,5)-triphosphate causes release of Ca^{2+} from intracellular stores and Ca^{2+} influx through voltage sensitive channels within the plasma membrane (Haisenleder et al., 1997; Willars et al., 2001), whereas DAG activates PKC and mitogen activated protein kinase (MAPK) pathways (Figure 2.4; Lin and Conn, 1999; Cheng et al., 2000b; Chamson-Reig et al., 2003). In addition to activation of PKC, DAG can cause an increase in the influx of Ca^{2+}, whereas IP_3 increases cytosolic Ca^{2+} from intracellular stores (Figure 2.4; Millar, 2005). Pituitary cell cultures treated with increased amounts of pertussis toxin, a protein based exotoxin, decreased inositol phosphate (IP) turnover, whereas decreased amounts of pertussis toxin did not decrease IP levels in response to GnRH. This suggested that a pertussis toxin sensitive G-protein couples with the receptor to initiate IP turnover (Hawkes et al., 1993). In addition, it was determined that Ca^{2+} entry and PKC activation were both involved in mediating GnRH

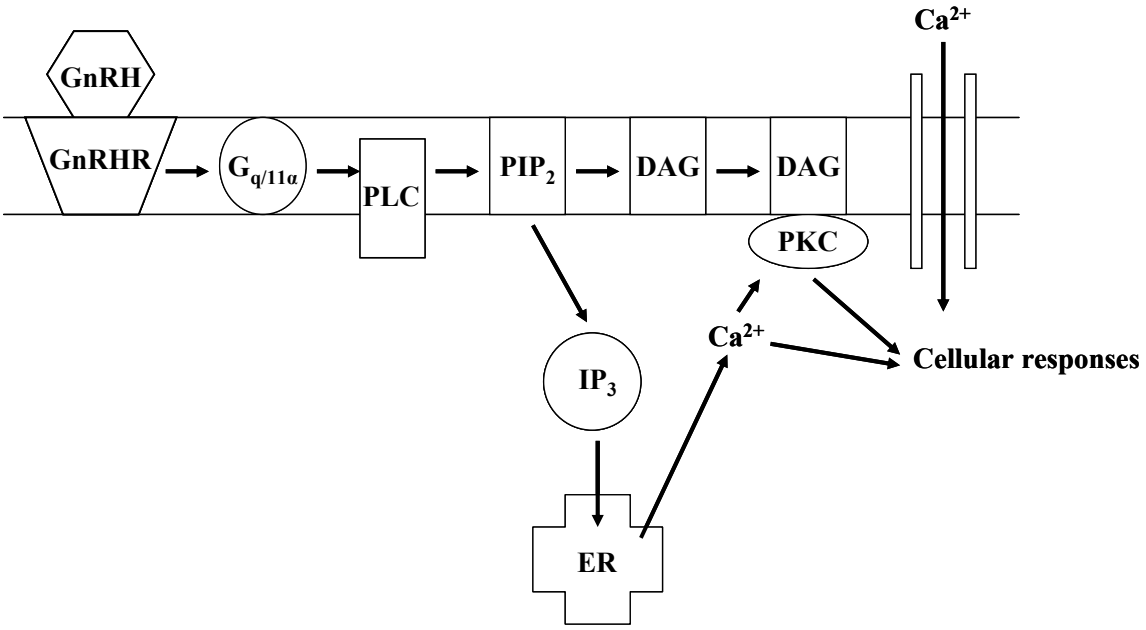


Figure 2.4. Activation of the protein kinase C (PKC) pathway following GnRH stimulation. Binding of GnRH to its receptor promotes coupling of GnRHR with $G_{q/11\alpha}$ which leads to activation of a classical signaling cascade, the PKC pathway. The G protein complex, $G_{q/11\alpha}$, activates phospholipase C (PLC) which hydrolyzes phosphatidylinositol (4,5)-bisphosphate (PIP_2) to generate the second messengers, inositol (1,4,5)-triphosphate (IP_3) and (1,2)-diacylglycerol (DAG). Inositol (1,4,5)-triphosphate controls Ca^{2+} release from the endoplasmic reticulum (ER), whereas DAG promotes influx of external Ca^{2+} and activates PKC pathways. Adapted from Kaiser et al. (1997).

stimulation of the human glycoprotein hormone α-subunit gene in αT3-1 and LβT2 cells (Saunders et al., 1998; Fowkes et al., 2002). However, expression of the LHβ- and FSHβ-subunit genes is dependent on PKC activation alone following GnRH stimulation (Saunders et al., 1998).

Besides activation of PIP_2, phospholipase A_2 (PLA_2) and D (PLD) are activated approximately 1 to 2 minutes after GnRH binds to its receptor, which may help in the formation of late DAG activation (Shacham et al., 1999; Chamson-Reig et al., 2003). Phospholipase D is regulated by the same G-protein as PLC; however, PKC regulates both PLC and PLD by different mechanisms. Thus, a change from initial activation of PLC to continual activation of PLD is due to the phosphorylation of a G-protein (Liscovitch, 1992). Lastly, activation of PLA_2 by GnRH binding to GnRHR liberates free fatty acids and lysophospolipids and is frequently associated with receptor-mediated cell activation (Stojilkovic et al., 1994).

Protein Kinase A. Gonadotropin-releasing hormone binding to its receptor can result in coupling with the G-protein, G_s, activating the PKA pathway (Figure 2.5) or G_i, inhibiting the PKA pathway. The GnRHR undergoes a conformational change, causing dissociation of the α-subunit from the β- and γ-subunits. The α-subunit will stimulate activity of adenylate cyclase to produce increased amounts of cAMP. Next, cAMP will activate PKA which consists of two regulatory (R) subunits, which could be any heterodimeric combination of the following family members; RIα, RIβ, RIIα and RIIβ (Newton and Messing, 2006). Protein kinase A is also made up of two catalytic (C)

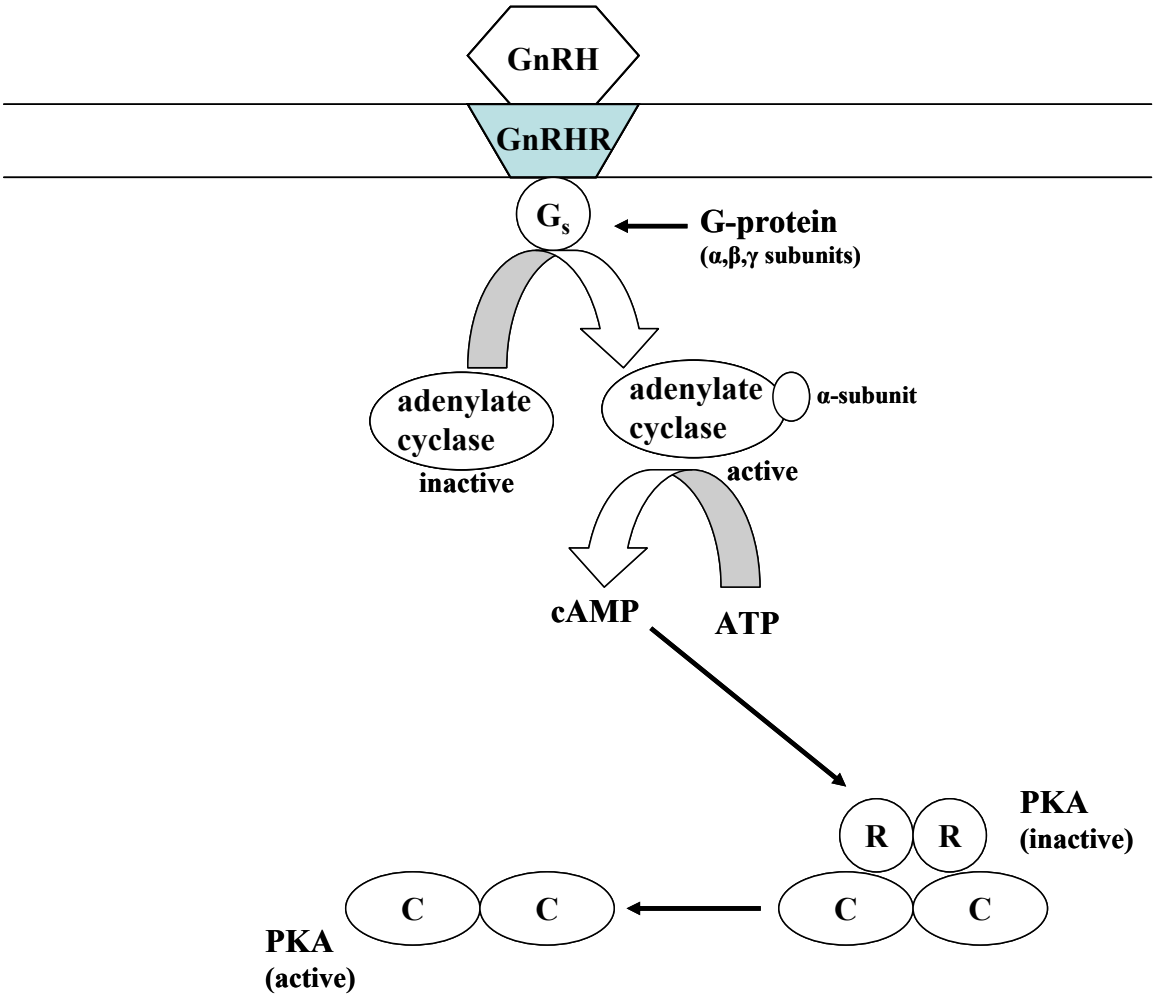


Figure 2.5. Activation of the protein kinase A (PKA) pathway following GnRH stimulation. Upon binding of GnRH to its receptor, the GnRHR can couple to the G-protein, G_s. This G-protein will then activate adenylate cyclase to produce cAMP. Cyclic AMP will activate PKA which is made up of two regulatory (R) and two catalytic (C) subunits in its inactive form. Once cAMP stimulates PKA this will dissociate the R subunits from the complex to activate the PKA pathway. Adapted from Kaiser et al. (1997).

subunits (Cα and Cβ). The α-subunits are expressed ubiquitously, whereas the β-subunits are not (Brandon et al., 1997). Garrel and associates (1995) cultured anterior pituitary cells and indicated that the direct activation of PKA and PKC changes the expression of genes encoding PKA RIIβ- and Cα-subunit isoforms. In addition, the activation of PKA or PKC alone regulates pituitary cell contents for the RI, RII and C subunits of PKA (Garrel et al., 1995). Upon activation by cAMP, the R subunits will dissociate from the C subunits, in turn activating the PKA pathway. The PKA pathway will contribute to various actions within the cell including transcription of the GnRHR gene (Naor et al., 2000; Millar et al., 2004).

The GnRHR has been shown to couple to G_s to activate the PKA pathway (Figure 2.5; Stanislaus et al., 1998). Long term treatment of rat somatolactotrope cells (GH3) with cAMP altered total cAMP-dependent protein kinase activity through effects on degradation of the catalytic subunit (Richardson et al., 1990). Sadie et al. (2003) indicated that GnRHR mRNA levels in αT3-1 cells are stimulated by 8-bromo-cAMP and forskolin. In addition, the mouse GnRHR promoter is regulated by PKA through a mechanism involving steroidogenic factor-1 (SF-1). Another indication that SF-1 is important in regulating PKA is that treatment with forskolin up-regulated SF-1 mRNA levels in αT3-1 cells. Additional studies indicated that primary pituitary cell cultures also utilize the PKA pathway upon GnRHR activation. Tsujii and associates (1995) reported that treatment of both pituitary and αT3-1 cells with pituitary adenylate cyclase-activating polypeptide (PACAP), increased α-subunit mRNA levels, implicating activation of GnRHRs by the cAMP pathway (Tsujii et al., 1995).

Mitogen-Activated Protein Kinases. *General.* Mitogen-activated protein kinases consist of protein serine/threonine kinases involved in processes which regulate cell growth, division and differentiation. Once activated, MAPKs are translocated to the nucleus, where they phosphorylate transcription factors as well as promote DNA synthesis and cell division (Han and Conn, 1999; Lin and Conn, 1999; Bonfil et al., 2004). There are generally 3 protein kinases involved in each MAPK cascade: MAPK, MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK). Four MAPK cascades are known in mammals: ERK1/2, JNK, p38 MAPK and ERK5/big MAPK (BMK; Naor et al., 2000; Bonfil et al., 2004; Morimoto et al., 2007). GnRH stimulates ERK, JNK and p38 MAPK in αT3-1, LβT2 and enhanced GnRHR transfected cells (GGH_3; Bonfil et al., 2004). Also, GnRH activates ERK5 in primary pituitary and αT3-1 cells (Naor et al., 2000). Studies have been done in these different cell lines to determine which MAPKs are activated following GnRH stimulation. For example, Reiss et al. (1997) treated αT3-1 cells with a GnRH agonist which resulted in the activation of MAPK by Ca^{2+}, protein tyrosine kinase (PTK), and PKC. Treatment with a GnRH agonist or PKC activator (TPA) resulted in a sustained response of MAPK activity. To date, all of the known MAPK cascades are activated by GnRH via a PKC-dependent mechanism (Figure 2.6; Levi et al., 1998; Naor et al., 2000).

ERK1/2. The most widely studied components of signal transduction pathways are ERK 1 and 2. Extracellular signal-regulated kinase 1/2 plays an important role in cell survival, motility and secretion. In addition, transactivation of the epidermal growth

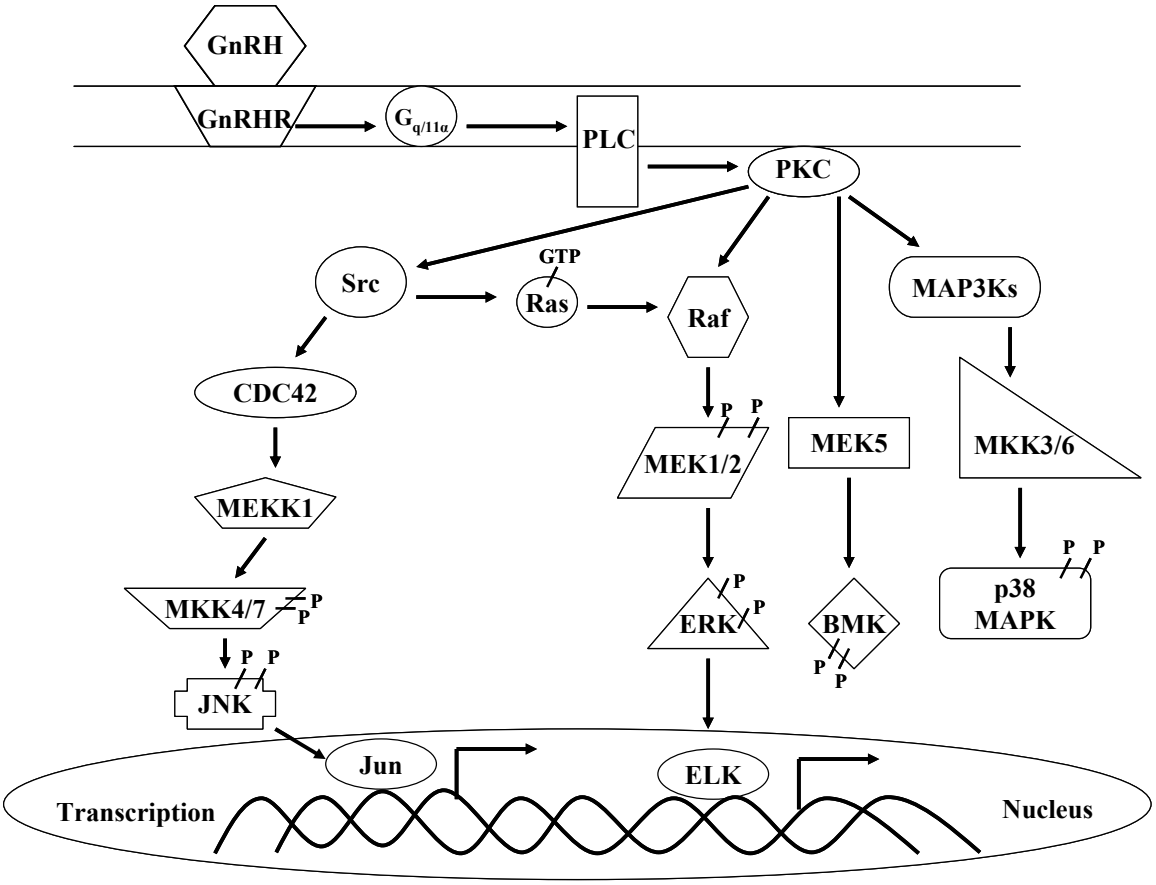


Figure 2.6. A general schematic of mitogen-activated protein kinase (MAPK) signaling cascades following GnRH binding to its cognate receptor. Adapted from Naor et al. (2000).

factor receptor is responsible for ERK1/2 responses due to the stimulation of specific GPCRs (Shah et al., 2003). Both ERK 1 and 2 are activated by protooncogene products and diverse extracellular stimuli that induce proliferation or enhance differentiation (Cobb and Goldsmith, 1995). A member of the Ras family of G-proteins activates Raf (MEKK) and MEK1/2, subsequently phosphorylating ERK1/2 (Figure 2.6; Bonfil et al., 2004). Gonadotropin-releasing hormone receptor mediates signaling via ERK in the *Xenopus* and human GnRHR. For example, *Xenopus* GnRHR mediates signaling to ERK by arrestins, whereas human GnRHR is less efficient than *Xenopus* GnRHR at causing MAPKs to translocate to the nucleus. This suggests that human GnRHR utilizes scaffolds other than arrestins to mediate ERK compartmentalization (Caunt et al., 2006).

Several mechanisms are involved in the attenuation and termination of ERK1/2 activation. Inhibitory and stimulatory responses determine the duration and strength of signals that start at the cell surface in response to ERK1/2 stimulation (Shah et al., 2003). Gonadotrope cells have been used to understand the mechanisms involved in ERK1/2 activation. For example, the Per1 gene is expressed in immortalized gonadotrope cell lines and is up-regulated by GnRH. This gene is activated by PKC and ERK1/2, representing a novel mechanism of GnRH signaling (Olcese et al., 2006). Additionally, activation of ERK1/2 and tyrosine phosphorylation in αT3-1 and wt28 [a clonal cell line consisting of GH3 (somatolactotrope) cells that were transfected with the mouse GnRHR and selected for prolactin/growth hormone release and IP production in response to GnRH; Johnson et al., 2000] cells differed in response to GnRH. Extracellular signal-regulated kinase 1/2 and tyrosine were activated in αT3-1 cells treated with the PKC

activator, phorbol 12,13-dibutyrate. However, the PKA activator, forskolin, stimulated ERK 1/2 phosphorylation in wt28 cells. An increase in tyrosine phosphorylation occurred following a treatment of the tyrosine phosphatase inhibitor, pervanadate, in wt28 cells. Treatment with both pervanadate and GnRH to both cell types resulted in an additive ERK 1/2 phosphorylation in αT3-1 cells, whereas ERK 1/2 phosphorylation was synergistic and sustained in wt28 cells. This suggests that the GnRHR may be activated through different cellular events depending on the tissue that confers its expression (Johnson et al., 2000).

Mitogen activated protein kinase pathways differ in cell lines from other tissues which have been used to study the relationship between GnRHR and ERK1/2. For example, expression of GnRHR in human embryonic kidney 293 cells is independent of Src and epidermal growth factor receptor transactivation due to GnRH-induced phosphorylation of ERK1/2. However, GnRHR is dependent on PKC in response to the activation of ERK1/2 which leads to constant activation and accumulation of ERK1/2 in the nucleus (Farshori et al., 2003; Shah et al., 2003). In addition to kidney cells, the activation of ERK1/2 through the PKC pathway is essential for GnRH-induced anti-proliferation of ovarian cancer cells (Kim et al., 2006).

JNK. Jun N-terminal kinase (stress-activated protein kinase; SAPK) is activated by members of the Ras family of G-proteins, MAPK/ERK kinase (MEKK1-4) and MAPK kinase (MKK4/7; Figure 2.6; Levi et al., 1998; Bonfil et al., 2004). In order to activate the transcription factors, c-Jun, activating transcription factor 2 (ATF2) and Elk1, the JNK cascade stimulates p21-activated kinase 1/mixed lineage kinase (PAK1/MLK),

MEKK1, MKK7, and JNK1/2. In response to GnRH, the JNK cascade involves PKC, c-Src family protein tyrosine kinase (PTK), cell division cycle 42 (CDC42/Rac1) and possibly MEKK1. As a result, these lead to JNK activation and c-Jun induction in response to GnRH in αT3-1 cells (Levi et al., 1998). In addition to αT3-1 cells, pituitary cell cultures from rats were treated with a JNK inhibitor or vehicle to determine if JNK directs gonadotropin subunit transcriptional responses to pulsatile GnRH (Haisenleder et al., 2008). The JNK inhibitor minimized both basal and GnRH-induced increases in FSHβ-subunit primary transcripts by half but had no effect on LHβ-subunit primary transcripts (Haisenleder et al., 2008). In addition, Xie et al. (2005) demonstrated that JNK and ERK work synergistically to regulate the human α-subunit transcriptional responses to GnRH. Besides its role in FSHβ- and α-subunit gene activation, JNK is also involved in GnRH signaling to genes encoding the GnRHR. GnRH regulation of the mouse GnRHR is conferred by an AP-1 binding site which binds JunD, FosB and c-Fos. Additionally, αT3-1 cells stably expressed with a dominant-negative c-Jun N-terminal kinase lost GnRH responsiveness. Thus, the interaction between GnRH and its cognate receptor is regulated by the JNK signaling cascade (Ellsworth et al., 2003b).

p38. One of the stress-activated protein kinases, p38 (SAPK2), is made up of four subunits, α, β, γ and δ (Bonfil et al., 2004). Similar to JNK, p38 MAPKs are activated by osmotic shock, heat shock, UV light, cellular stress, bacterial infection, pro-inflammatory cytokines and DNA-damaging agents. This stress-activated protein kinase modulates transcriptional activity, cell cycle, and programmed cell death in response to ligands that bind GPCRs as well as environmental stress and inflammatory cytokines. Activation of

p38 MAPKs occurs via the phosphorylation of a threonine at position 180 and a tyrosine in the activation loop positioned at 182 (Kim et al., 2004). Lastly, Haisenleder and associates (2008) treated pituitary rat cell cultures with a p38 inhibitor and indicated that p38 had no effect on LHβ- or FSHβ-subunit primary transcripts.

ERK5 (BMK). Extracellular signal-regulated kinase 5 (ERK5), also termed big MAPK (BMK), is aptly named because its C-terminus consists of a unique 400-amino acid extension. It plays an important role in biological pathways that modulate transcription. Extracellular signal-regulated kinase 5 is activated by hyperosmolarity, growth factors (EGF and nerve growth factor) and oxidative stress (Morimoto et al., 2007). Knockout mice for ERK5 died during embryonic stages due to angiogenic failure and cardiovascular defects (Regan et al., 2002; Sohn et al., 2002; Yan et al., 2003). Hayashi and associates (2004) deleted ERK5 in adult mice which led to death due to leakage in blood vessels and hemorrhages in multiple organs which caused apoptosis in endothelial cells. This suggested that ERK5 is responsible for endothelial cell survival and maintenance of blood vascular integrity. Others indicated that ERK5 plays two important roles in gene expression; activation of transcription factors by direct phosphorylation and enhancement of transcriptional activation activity by autophosphorylation of 2 C-terminal regions (Morimoto et al., 2007). Besides its PKC dependency, the involvement of ERK5 in the regulation of gonadotropin subunits and GnRHR synthesis as well as whether or not it translocates to the nucleus is still unclear (Naor et al., 2000).

Hormonal Regulation

GnRH. In order to maintain normal reproductive function, GnRH is a key regulator of its own receptor. Gonadotropin-releasing hormone can up-regulate its receptor by increasing GnRHR mRNA levels (transcriptional regulation) or altering receptor numbers post-translationally (Cheng and Leung, 2000). For example, pulsatile GnRH at a low concentration caused an up-regulation of GnRHRs, whereas continuous GnRH at a high concentration resulted in down-regulation and desensitization of GnRHRs (Loumaye and Catt, 1982; Katt et al., 1985). Receptor numbers at the cell surface may influence the rate of receptor biosynthesis by regulation at the transcriptional level. However, it may also be regulated at the post-translational level by recycling, modification, or degradation of the receptor (Kaiser et al., 1993). Kaiser and associates (1993) detected that treatment of rat primary pituitary cultures with continuous GnRH did not alter GnRHR mRNA levels. However, administration of pulsatile GnRH resulted in increased GnRHR mRNA levels. These studies *in vitro* revealed that there is homologous regulation of GnRHR gene expression (Kaiser et al., 1993).

A study in wethers indicated that daily administration of a potent GnRH agonist decreased GnRHR mRNA levels (Wu et al., 1994). Adams and associates (1996) also detected that administration of high concentrations of GnRH induced a down-regulation of GnRHR mRNA levels in wethers. Recently, it was reported that administration of continuous GnRH to primary rat anterior pituitary cells for 6 hours increased the levels of GnRHR mRNA (Cheon et al., 2000). The number of GnRHRs on gonadotrope cells varied due to fluctuations in GnRH secretion during the estrous cycle in the rat. During

proestrus in the female rat, GnRHR mRNA levels, receptor numbers and sensitivity to GnRH were highest within the anterior pituitary gland (Bauer-Dantoin et al., 1993). Further, the anterior pituitary gland contained increased GnRHR mRNA levels during the follicular phase in the ewe due to sensitivity of GnRH prior to the preovulatory gonadotropin surge (Turzillo et al., 1994). In addition, Cheon et al. (2000) used a GnRH agonist to block transcriptional activity of the GnRHR gene. Therefore, this suggests that homologous up-regulation of GnRHR numbers may occur through transcriptional activation of the gene versus mRNA stability (Cheon et al., 2000).

Borgeat and associates (1972) treated anterior pituitary tissue of male rats with a GnRH agonist which increased the intracellular concentrations of cAMP and also released the gonadotropins, LH and FSH. However, αT3-1 cells treated with a GnRH agonist activated PKC pathways, whereas there was no significant change in cAMP levels (Horn et al., 1991). This mechanistic difference may be due to testosterone because intact male rats treated with a GnRH agonist increased cAMP levels. In contrast, significant alterations in cAMP levels have not been detected in ovine primary pituitary cells following GnRH treatment (Kaiser et al., 1997). Huckle and associates (1988) indicated that GnRH is regulated by Ca^{2+} and PKC in rat primary pituitary cultures due to an increase in GnRHRs. However, PKC activators can stimulate the synthesis of GnRHR by a mechanism independent of GnRH-induced up-regulation (Braden et al., 1991).

Gonadotropin-releasing hormone receptor promoter activity increased when αT3-1 cells were treated with a GnRH agonist, whereas treatment with a GnRH antagonist reduced mouse GnRHR promoter activity (Norwitz et al., 1999; White et al., 1999).

Treatment with the PKC activator, phorbol-12-myristate-13-acetate (PMA), increased mouse GnRHR promoter activity, whereas the PKC inhibitor, GF109203X, blocked promoter activity, indicating that activation of the PKC pathway is important for GnRHR promoter activity. However, αT3-1 cells treated with the PKA activator, forskolin, did not affect GnRHR promoter activity (White et al., 1999). In contrast, transfection of the mouse GnRHR gene promoter into GGH_3 cells (GH_3 cells stably expressing GnRHR) treated with GnRH or a GnRH agonist (buserelin), stimulated promoter activity and increased cAMP release (Lin and Conn, 1998). Similarly, treatment with an adenylate cyclase inhibitor, SQ22536, significantly decreased buserelin-activated GnRHR promoter activity. Therefore, GnRH regulation of GnRHRs in the GGH_3 cell line occurs via both cAMP and PKC pathways (Lin and Conn, 1998). Two laboratories, (Norwitz et al. (1999; White et al., 1999) identified an AP-1 element critical for responsiveness of the mouse GnRHR gene to GnRH. In addition, homologous regulation of GnRHR involve PKC activation of the JNK signaling cascade, resulting in the binding of JunD, FosB and c-Fos to the AP-1 element (Ellsworth et al., 2003b).

Gonadotropic Hormones. The gonadotropic hormone, LH, as well as its agonist, hCG, can have an effect on GnRHR mRNA levels. Li et al. (1996) indicated that treatment of GT1-7 neurons with hCG down-regulates expression of the GnRHR gene by decreasing stability of transcripts. Therefore, LH/hCG may disrupt the self-stimulatory mechanism of GnRH synthesis and secretion of hypothalamic neurons by the down-regulation of GnRHR (Li et al., 1996). Additionally, treatment of human granulosa-luteal

cells with hCG decreased GnRHR mRNA levels without altering the expression of the GnRH gene (Peng et al., 1994). Similarly, Cheng and Leung (2000) detected a decrease in GnRHR mRNA levels in rat granulosa cells following treatment with LH.

Botte et al. (1999) treated adult male rats with LH and FSH agonists (hCG and h-rec FSH). There was no significant alteration in GnRH or GnRHR mRNA levels following treatment with h-rec FSH in the rat testis (Tapanainen et al., 1993; Botte et al., 1999). However, hCG, a glycoprotein that binds to LH receptors, decreased GnRHR mRNA levels in the rat testis. Luteinizing hormone receptors, GnRHRs and testosterone secretion had been detected in Leydig cells. In addition, LH regulates GnRHR mRNA levels within Leydig cells. In contrast, the lack of an FSH effect on GnRHR mRNA amounts may be due to the absence of FSH receptors on Leydig cells (Botte et al., 1999). Following testosterone treatment, GnRHR mRNA levels increased, an effect that may be associated to negative feedback of testosterone on LH release (Botte et al., 1999). In addition, LH increased following treatment with a GnRH agonist or hCG, resulting in decreased GnRHR mRNA levels. Therefore, this study suggested that LH down-regulates GnRHR mRNA stability and gene expression in the rat testis (Botte et al., 1999).

Steroid Hormones. *General.* Steroid hormones play an important role in the reproductive axis. The secretion of steroid hormones by the gonads elicits negative or positive effects on gonadotrope cells within the anterior pituitary gland, regulating the

production of GnRHRs. The steroid hormones, E_2, glucocorticoids, P_4 and testosterone, have been shown to regulate levels of GnRHR mRNA and receptor numbers.

Estradiol-17β. Studies involving rat primary pituitary cultures indicated that treatment with E_2 increased the number of GnRHRs. Estradiol-17β-mediated GnRHR induction may involve new protein synthesis (Menon et al., 1985). Emons and associates (1988) indicated that short-term treatment with E_2 caused a significant decrease in GnRH-induced, LH release that was potentially due to a reduction of available GnRHRs. Kaiser et al. (1993) studied the regulation of GnRHR numbers at the pre-translational level. Ovariectomized rats had increased GnRHR mRNA levels; however, replacement therapy with E_2 significantly decreased GnRHR mRNA levels in ovariectomized compared to control rats. This suggested that GnRHR synthesis was altered at the pre-translational level due to differences in hypothalamic GnRH secretion (Kaiser et al., 1993).

Hypothalamic-pituitary disconnection in ovariectomized ewes resulted in decreased GnRHR numbers within the anterior pituitary gland, mainly due to the lack of GnRH stimulation (Clarke et al., 1987; Gregg and Nett, 1989; Turzillo et al., 1995), whereas GnRHR mRNA levels were not altered in controls. This suggested that GnRHR mRNA levels in ovariectomized ewes do not require continuous stimulation by GnRH (Turzillo et al., 1995). One reason for this may be that there are minor alterations at the transcriptional level. However, more modifications occur during post-transcriptional events, resulting in changes in GnRHR numbers. The situation may be similar in αT3-1 cells because GnRH agonist treatment significantly increased GnRHR numbers, whereas

GnRHR mRNA levels did not change (Tsutsumi et al., 1993; Mason et al., 1994). Crowder and Nett (1984) suggested that E_2 stimulated an increase in the number of GnRHRs, possibly contributing to the preovulatory LH surge in ewes. Gregg and Nett (1989) demonstrated that E_2 can independently increase GnRHR numbers following hypothalamic-pituitary disconnection in the ewe. Similarly, Turzillo et al. (1995) reported that E_2 increased GnRHR RNA levels using the same model system.

Increased GnRHR mRNA concentrations were detected following treatment with E_2 in ovariectomized ewes (Gregg and Nett, 1989; Moss et al., 1981; Clarke et al., 1988; Turzillo et al., 1994; 1995; Hamernik et al., 1995). Gonadotropin releasing hormone receptor mRNA levels and receptor numbers in ovariectomized ewes treated with both GnRH and E_2 did not differ from saline injected controls. However, GnRHR mRNA levels and receptor numbers were significantly increased in ewes treated with both GnRH and E_2 compared to ewes treated with GnRH alone. This suggested that continuous administration of GnRH resulted in a lower baseline for GnRH numbers and mRNA levels (Turzillo et al., 1998). A study in ovariectomized ewes following hypothalamic-pituitary disconnection indicated that a low amount of E_2 and GnRH are required to significantly increase GnRHR mRNA levels and receptor numbers. It was concluded that there is a synergistic interaction between E_2 and GnRH in the regulation of GnRHR gene expression (Kirkpatrick et al., 1998a). Others also reported that continuous treatment with E_2 increased pituitary GnRHR numbers *in vivo* both in ovariectomized sheep (Moss et al., 1981) and cows (Schoenemann et al., 1985) and *in vitro* in rat (Menon et al., 1985) and ovine (Laws et al., 1990b; Gregg et al., 1990; Wu et al., 1994) pituitary cell cultures.

Glucocorticoids. In addition to E_2, glucocorticoids can stimulate GnRHR gene expression. Administration of dexamethasone to GnRH deficient mice resulted in a significant stimulation of GnRHR gene expression (Rosen et al., 1991). Additionally, the glucocorticoid antagonist, RU486, blocked transcriptional activity of the mouse GnRHR promoter (Maya-Nunez and Conn, 2003). Further investigation revealed a putative glucocorticoid response element (GRE) within the mouse GnRHR promoter located between -331/-225 bp of 5' flanking region. In addition to dexamethasone responsiveness, this region contains important elements for basal and buserelin-stimulated promoter activity. Mutation of an AP-1 element located within this region resulted in enhanced functional activity of the mouse GnRHR promoter following treatment with a GnRH agonist or dexamethosone. Therefore, the dexamethosone mechanism that altered GnRHR mRNA levels may be due to direct stimulation of the AP-1 binding proteins within the mouse GnRHR promoter (Maya-Nunez and Conn, 2003). Additionally, glucocorticoid repression of transcription in the hypothalamic cell lines, GT1-3 and GT1-7, suggested that glucocorticoid receptor acts directly within GnRH neurons. This may be responsible for the negative feedback effect of glucocorticoids in the reproductive axis (Chandran et al., 1994).

Progesterone. In addition to glucocorticoids, GnRHR is regulated by P_4. For instance, P_4 treatment of ovine pituitary and αT3-1 cells *in vitro* as well as P_4 delivery to sheep *in vivo* reduced levels of GnRHR mRNA (Wu et al., 1994). Bauer-Dantoin and associates (1995) indicated that P_4 did not significantly alter GnRHR mRNA levels prior to or during the E_2-induced LH surge in rats. However, treatment with P_4 decreased

GnRHR mRNA levels at the end of the LH surge in comparison to levels in animals treated with only E_2. It was concluded that preovulatory P_4 secretion may contribute to the decreased GnRHR mRNA levels that occur with the decline in gonadotropin levels (Bauer-Dantoin et al., 1995). Progesterone also regulates the GnRHR gene in the human. The human GnRHR gene promoter contains a putative progesterone response element (PRE; Fan et al., 1995). The physiological effects of P_4 are controlled by a specific nuclear receptor protein, progesterone receptor (PR), which has two different isoforms, PR-A and PR-B. These isoforms play different roles in the transcriptional regulation of the human GnRHR within the pituitary and placenta (Cheng et al., 2001a).

Testosterone. Numerous steroid hormones affect GnRHR numbers and mRNA levels. Compared to other steroid hormones, however, testosterone plays a reduced role in the regulation of GnRHRs. Kaiser and associates (1993) detected increased GnRHR mRNA levels following castration of rats. Replacement therapy with testosterone propionate moderately decreased GnRHR mRNA levels compared to control rats. However, replacement with E_2 in ovariectomized female rats resulted in a significant decrease in GnRHR mRNA levels. This suggested that pituitary GnRHR mRNA levels are due to alterations in the pattern of hypothalamic GnRH secretion (Kaiser et al., 1993). In contrast, Botte et al., (1999) detected that hCG treatment decreased GnRHR mRNA levels in the testis. However, testicular GnRHR mRNA levels were increased by testosterone treatment. It was suggested that this stimulation was mediated through a reduction in LH secretion which may be due to the negative feedback of testosterone.

Activin/Follistatin. Activin and follistatin are members of the transforming growth factor-β superfamily. They control many vital physiological processes important for development, growth, and functional integrity of tissues, such as the pituitary (Bilezikjian et al., 2006). Activin is produced by pituitary cells, including gonadotropes, to help maintain a normal reproductive axis. It has the ability to stimulate FSH secretion from gonadotropes within the anterior pituitary gland. Follistatin, also produced in the anterior pituitary gland, is a glycoprotein that binds to activin itself, inhibiting the synthesis and release of FSH from the pituitary (Bilezikjian et al., 2006). Overexpression of follistatin is linked to infertility, possibly due to a disruption of activin and bone morphogenetic protein (BMP) signals at the level of both the gonads and pituitary (Guo et al., 1998).

The gonadotrope derived-cell lines, LβT2 and αT3-1, activate mechanisms correlated with the actions of activin in gonadotropes. Studies in these cell lines have revealed that both FSHβ-subunit and GnRHR promoters are modulated by activin (Fernandez-Vazquez et al., 1996; Duval et al., 1999; Pernasetti et al., 2001; Norwitz et al., 2002; Suszko et al., 2003; Bernard, 2004). The αT3-1 cell line, which endogenously produces activin, was used to study gonadotrope expression of the mouse GnRHR gene promoter (Duval et al., 1999). Activin responsiveness is mediated by the regulatory element GnRHR activating sequence (GRAS) in the mouse GnRHR gene (Ellsworth et al., 2003a). Follistatin blocked activin responsiveness of the mouse GnRHR gene suggesting that activin stimulates gonadotropes by an autocrine/paracrine manner (Duval et al., 1999). A downstream activin regulatory element (DARE) comprises a unique and

complex activin/TGF-β responsive enhancer in the promoter of the mouse GnRHR gene. Ellsworth and associates (2003) identified that Smad 3 and 4, AP-1 and a forkhead DNA binding protein, FoxL2 bind to the GRAS element. Both GRAS and DARE are involved in activin responsiveness of the mouse GnRHR gene and may be both structurally and functionally coupled (Cherrington et al., 2005). Therefore, activin and follistatin depend on one another to regulate FSH suppression or secretion in gonadotrope cells of the anterior pituitary gland.

Inhibin. Inhibin, a peptide that binds to activin receptors, is a member of the transforming growth factor-β (TGF-β) superfamily that, much like follistatin, inhibits FSH synthesis and secretion from the anterior pituitary (Ethier and Findlay, 2001; Gregory and Kaiser, 2004). Inhibin also regulates other pituitary functions such as the synthesis of GnRHRs by gonadotropes (Braden et al., 1990; Fernandez-Vazquez et al., 1996). Inhibin up-regulated GnRHR mRNA levels in ovine pituitary cell cultures (Laws et al., 1990a; Wu et al., 1994). Additional studies utilizing ovine pituitary cell cultures indicated that E_2 and inhibin may have additive effects on the up-regulation of GnRHR numbers (Gregg et al., 1991). However, treatment of rat primary pituitary cultures with inhibin decreased GnRHR numbers (Wang et al., 1988) and suppressed GnRH-induced up-regulation of GnRHR binding sites (Wang et al., 1989). Therefore, inhibin utilizes different mechanisms in the rat and sheep.

Transcriptional Regulation

General. The transcriptional activity of the GnRHR gene is regulated by a variety of transcription factors in mammalian species such as the human, mouse and rat (Figure 2.7). Transcription factors including GRAS, Oct-1, LIM-related factors, SF-1, AP-1, GATA, nuclear factor-κB (NF-κB), specificity protein (Sp) factors and PR play an important role in controlling the transcriptional activity of the GnRHR gene and will be further described within this section. The human GnRHR gene promoter contains several placenta specific elements located between -1718 and -1509 bp of proximal promoter; an Oct-1, cAMP response element (CRE), GATA and AP-1 element (Figure 2.7; Cheng et al., 2001b). The human GnRHR gene promoter also contains another AP-1 element regulated by GnRH located at -1000/-994 bp 5' flanking region and a progesterone response element (PRE; Figure 2.7). Additionally, the human GnRHR promoter contains two putative C/EBP motifs and a GATA binding site that are granulosa-luteal cell-specific (SVOG-4o and SVOG-4m; Figure 2.7; Cheng et al., 2002a). Lastly, the human GnRHR gene promoter contains several cell specific elements downstream including AP/CRE -1, AP/CRE-2, SF-1 and AP-1 (Figure 2.7). The mouse GnRHR promoter is also regulated by several elements. Gonadotrope specific activity is conferred by a tripartite basal enhancer consisting of SF-1 (-244/-236), AP-1 (-336/-330) and a novel element termed GRAS (-391/-380) binding sites (Figure 2.8; Duval et al., 1997a). Approximately 60% of promoter activity was lost when each element was mutated individually, whereas about 80% of promoter activity was lost when 2 elements

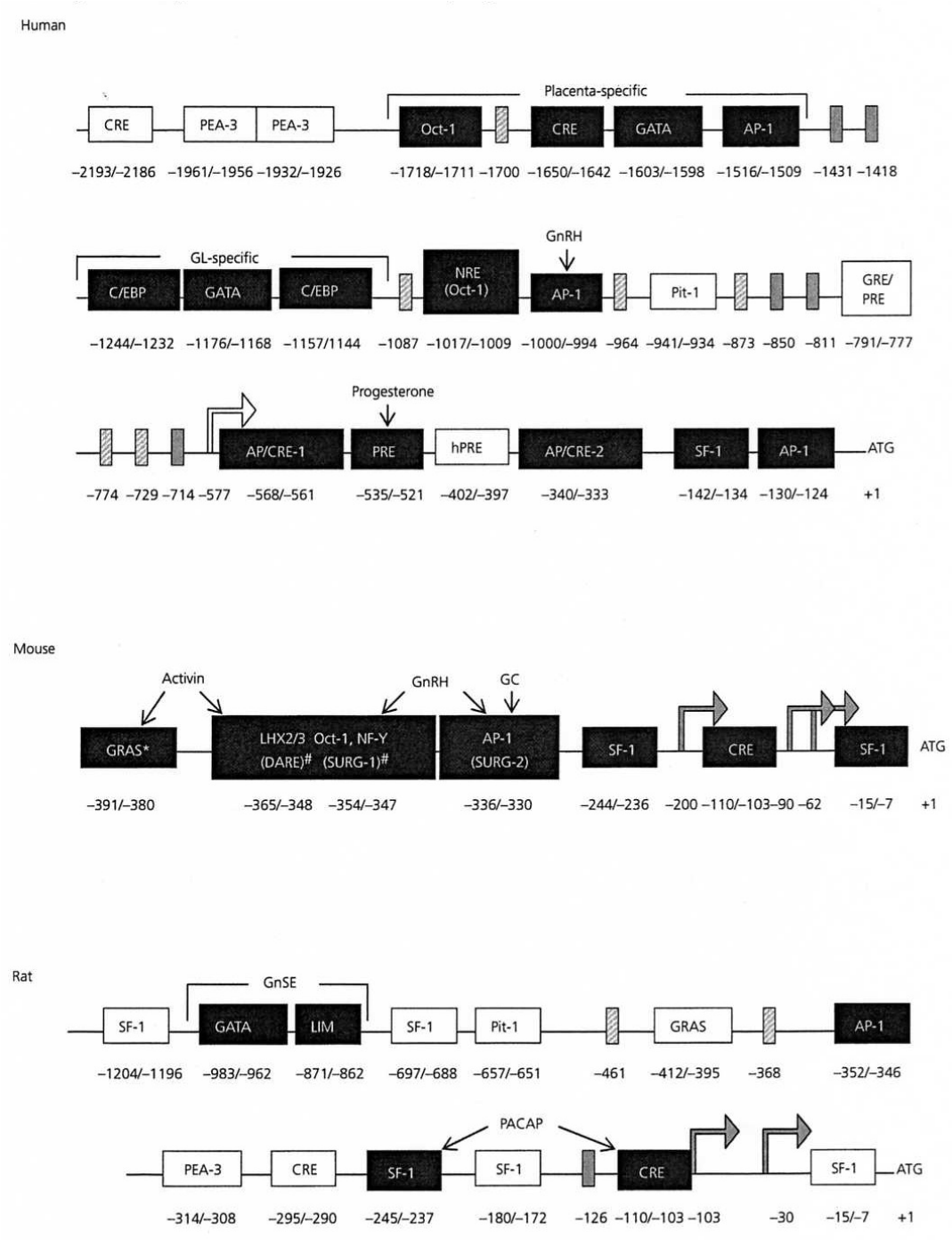


Figure 2.7. Schematic representation of elements conferring basal and hormonal regulation of the GnRHR gene promoter in the human, mouse and rat. The striped and shaded boxes represent CCAAT and TATA boxes, respectively. Black boxes indicate binding sites that have been functionally characterized, whereas white boxes identify putative binding sites. Transcription start sites are represented by arrows and translational start sites are indicated with an 'ATG'. From Hapgood et al. (2005).

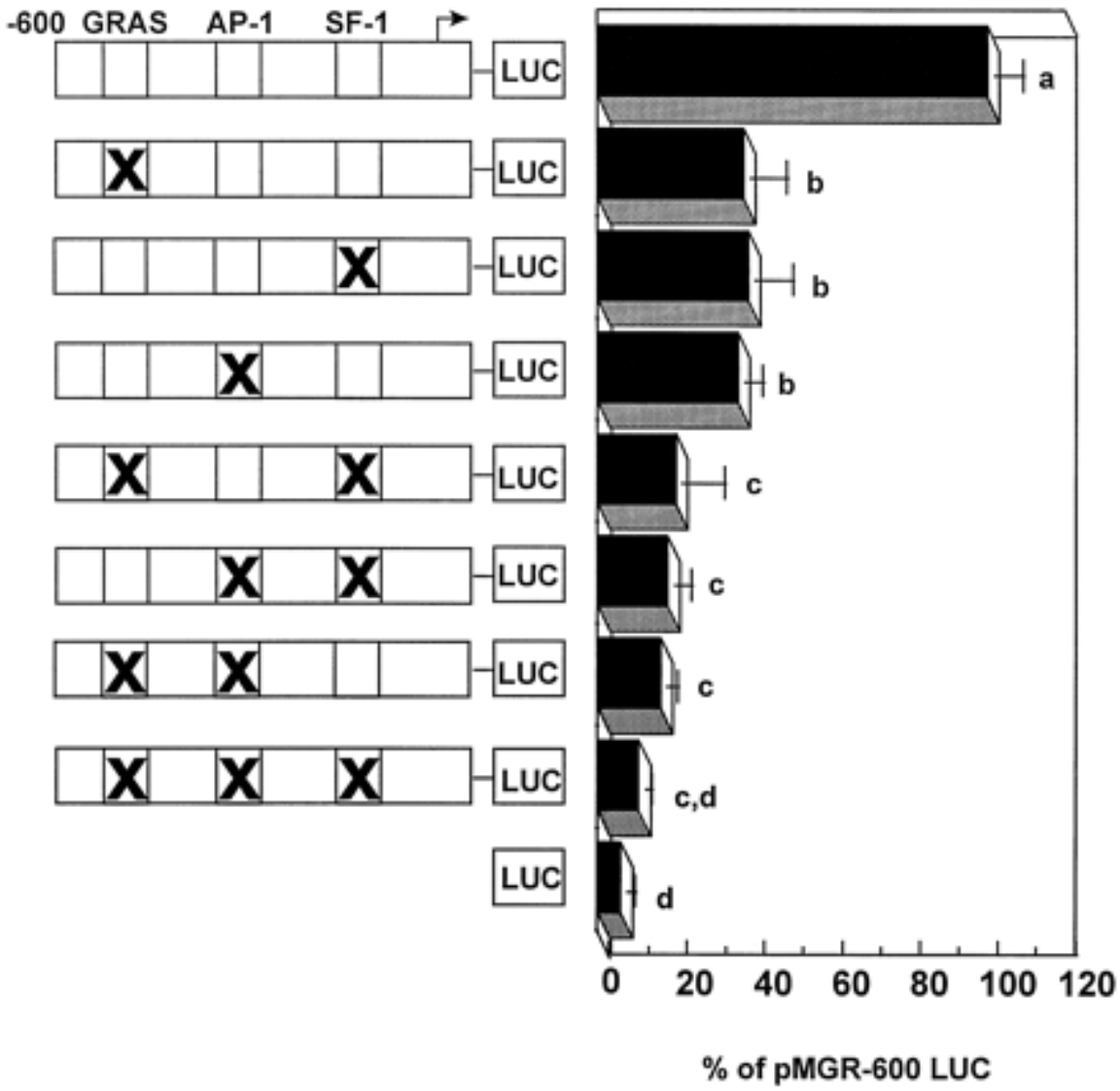


Figure 2.8. Cell-specific expression of the mouse GnRHR gene is regulated by a tripartite enhancer which consists of 3 elements; steroidogenic factor-1 (SF-1), activating protein-1 (AP-1) and GnRH receptor activating sequence (GRAS). Transient transfections were performed using luciferase (LUC) reporter vectors containing single, double, or triple mutations of the elements or promoterless control of -600 bp 5' flanking region of the mouse GnRHR gene promoter in αT3-1 cells. Cells were harvested 24 hours following transfection and assayed for both LUC and β-galactosidase (β-gal) activity. Luciferase values were divided by β-gal values to adjust for transfection efficiency. Differences are indicated by the bars with different letters ($P < 0.05$). From Duval et al. (1997a).

were mutated together. Finally, promoter activity was reduced to that of promoterless control when all 3 elements were mutated within the tripartite enhancer (Figure 2.8; Duval et al., 1997a). Additionally, LHX2/3, Oct-1, nuclear factor-Y (NF-Y), DARE and SURG-1 regulate both activin and GnRH responsiveness within the mouse GnRHR gene promoter (Figure 2.7; Norwitz et al., 2002; Ellsworth et al., 2003a). Lastly, a CRE in the mouse promoter is critical for basal activity in LβT2 and GGH3 cells (Figure 2.7; Maya-Nunez et al., 1999). The rat GnRHR gene promoter also contains the tripartite enhancer previously described in the mouse (Pincas et al., 2001). However, the rat promoter is regulated by a GnRHR specific enhancer (GnSE) that contains GATA and LIM binding sites. The GnSE helps confer gonadotrope specific expression via its interaction with an SF-1 element located at -275/-266 bp of proximal promoter (Figure 2.7; Pincas et al., 1998; 2001). Therefore, the human, mouse, and rat GnRHR promoters are regulated by multiple binding factors, many of which are unique to each species (Figure 2.7; Hapgood et al., 2005).

GnRH Receptor Activating Sequence. GnRH receptor activating sequence was identified as part of a tripartite enhancer that regulates cell-specific expression of the mouse GnRHR gene in the gonadotrope-derived αT3-1 cell line (Duval et al., 1997a) and work synergistically with an AP-1 and SF-1 binding site. In addition, GRAS regulates activin responsiveness of the mouse GnRHR gene promoter (Ellsworth et al., 2003a). The functional activity of GRAS is dependent on the organization of a multi-protein complex which is made up of Smad4, Smad3, AP-1, and a member of the forkhead

family of DNA binding proteins, FoxL2. Using 2 bp transversion mutations within the GRAS element, a Smad4 interaction at the 5' end of GRAS was revealed. This allowed for AP-1 and/or FoxL2 to bind to the remaining sequence. Furthermore, mutation of either the 5' Smad binding site or a putative forkhead binding site located at the 3' end of the element does not allow for the activation of FoxL2 at GRAS (Ellsworth et al., 2003a). Overall, GRAS is an important regulator of both basal activity and activin responsiveness of the mouse GnRHR gene promoter.

Octamer Transcription Factor-1. Octamer transcription factor-1 belongs to the POU family of transcription factors. It binds to target sequences through the POU family's bipartite DNA-binding domains (Sturm et al., 1988; Scheidereit et al., 1988; Muller et al., 1988). Octamer transcription factor-1 is essential for the transcription of both mRNA and small nuclear RNA-type RNA polymerase II promoters. Therefore, the promoter-specific activation domains of Oct-1 serve in combination with specific coactivators to stimulate gene expression (Yang et al., 1991; Tanaka et al., 1992; Pfisterer et al., 1994; Strubin et al., 1995; Gstaiger et al., 1995). Additionally, Oct-1, can recruit transcription factor IIB (TFIIB), critical to basal transcriptional machinery, to the initiation site to enhance activity (Nakshatri et al., 1995).

The human GnRHR contains an Oct-1 binding site located at -1017/-1009 bp of proximal promoter, which was originally referred to as a negative regulatory element (NRE; Cheng et al., 2002b). Deletion of the 3' end of the human GnRHR promoter revealed that the repressive activity of the NRE could be isolated to a putative Oct-1

regulatory sequence. Transient transfection studies indicated that Oct-1 displays repressive effects on the human GnRHR promoter in ovarian (OVCAR-3; Kang et al., 2000), placental (JEG-3; Cheng et al., 2001b), gonadotrope-derived αT3-1 (Kang et al., 2000) and granulosa-luteal cells (Cheng et al., 2002b). The transcription factor, Oct-1, may play an important role in silencing GnRHR gene transcription across species. The repressive role of the Oct-1 sequence may be evolutionarily conserved because alteration of the Oct-1 sequence in the rodent GnRHR promoter by a single bp did not disable its silencing activity (Cheng et al., 2002b).

Octamer transcription factor-1 can also have a positive effect on the GnRHR gene promoter. The mouse GnRHR gene promoter contains a cis-regulatory element designated Sequence Underlying Responsiveness to GnRH 1 (SURG-1; Norwitz et al., 1999) that is stimulated upon treatment with GnRH (Kam et al., 2005). Three elements have been identified within SURG-1; NF-Y, Oct-1 and AP-1. Mutations of each element alone decreased stimulation following treatment with a GnRH agonist (Norwitz et al., 1999; Kam et al., 2005). However, a mutation which consisted of both Oct-1 and AP-1 resulted in reduced basal transcriptional activity and abolished GnRH stimulation (Kam et al., 2005). In addition, Oct-1 small interfering RNA reduced mouse GnRHR promoter activity, confirming that Oct-1 is critical for gene expression. Therefore, NF-Y and Oct-1 bind to SURG-1 to direct basal and GnRH-stimulated expression of the mouse GnRHR gene (Kam et al., 2005).

LIM-Related Factors. The LIM-homeodomain (LIM-HD) proteins are a subfamily of proteins consisting of a C-terminal DNA binding homeodomain and 2 N-terminal, cysteine-rich, zinc finger-like LIM motifs that mediate protein-protein interactions (Jurata et al., 1998; Bach, 2000). To date, there are at least 12 LIM-HD genes that encode developmental regulatory proteins (Bach, 2000). LIM related factors play important roles in cell lineage specification and are involved in many developmental pathways. This family of proteins consists of Lhx2, Lhx3, Lhx4, Isl-1, Isl-2 and Lmx-1 (Roberson et al., 1994). The majority of these factors (Is1-1, Isl-2, Lhx2, Lhx3 and Lhx4) have been implicated in pituitary development (Mullen et al., 2007). The LIM-homeodomain transcription factors, Lhx3, Lhx4 and Isl-1 are important during the early developmental stages within the anterior pituitary gland (Pfaff et al., 1996) and Lhx3 and Lhx4 are also involved in the development of the nervous system (Mullen et al., 2007). In addition, Lhx3 is critical during gonadotrope differentiation (Sheng et al., 1996). Furthermore, these factors are important for gonadotrope-specific expression of the rat GnRHR gene (Granger et al., 2006).

Pincas and associates (2001) identified a GnSE located at -1135/-753 bp in the rat GnRHR promoter. Response elements located within the GnSE (-983/-962 and -871/-862) interact with GATA and LIM-related factors, respectively, indicating the importance of these transcription factors in regulation of the rat GnRHR gene (Pincas et al., 2001). Recently, it has been indicated that the interaction between Isl-1 and Lhx3 with SF-1 activates both the rat and human GnRHR promoters, suggesting that this combination of transcription factors is evolutionarily conserved among mammals (Granger et al., 2006).

Deletion of the LIM response element decreased rat and human GnRHR promoter activity in CHO cells. Finally, a transgenic model demonstrated that the overlapping expression of Isl-1 and Lhx3 in the developing pituitary is associated with GnRHR promoter activity (Granger et al., 2006).

Steroidogenic Factor-1. Steroidogenic factor-1 is an orphan member of the nuclear hormone receptor family that is essential for mammalian gonadogenesis prior to sexual differentiation and for normal endocrine development and function. Nuclear hormone receptors usually activate transcription as a homodimer or heterodimer, whereas SF-1 activates gene expression in a monomeric form (Nachtigal et al., 1998; Li et al., 1999). Steroidogenic factor-1 has a zinc finger motif which is involved in DNA binding and an Ftz-F1 box, a region downstream of the zinc finger motif, that assists binding of SF-1 to specific DNA sequences. In addition, SF-1 binds to a gonadotrope specific element (GSE; De Santa Barbara et al., 1998; Li et al., 1999).

A GSE motif within the human GnRHR gene promoter is located at -142/-134 bp of 5' flanking region and mediates cell-specific expression. Expression of the antisense SF-1 mRNA reduced human GnRHR promoter activity in αT3-1 cells, whereas activity in human ovarian adenocarcinoma (SKOV-3) and monkey kidney (COS-7) cells remained unaffected (Ngan et al., 1999). In αT3-1 cells, GnRHR promoter activity is conferred by SF-1 binding sites in other species such as the mouse (Duval et al., 1997a, 1997b), rat (Pincas et al., 2001), sheep (Duval et al., 2000), and pig (Cederberg et al., unpublished data). A GSE also plays an important role in the cell-specific expression of

the glycoprotein α- and LHβ-subunit genes, suggesting an important role for GSEs in the regulation of genes within the anterior pituitary gland (Horn et al., 1992). Additionally, regulation of the equine LHβ-subunit gene in LβT2 cells is controlled by 2 SF-1 elements (Wolfe, 1999). Furthermore, analysis of the mouse FSHβ-subunit gene in LβT2 cells is regulated by a critical interaction among elements, including 2 proximal GSEs and a downstream NF-Y element (Jacobs et al., 2003).

Steroidogenic factor-1 mRNA levels are increased in αT3-1 cells following treatment with forskolin, a cAMP activator (Sadie et al., 2003). Consistent with this, the mouse GnRHR gene promoter utilizes SF-1 to regulate responsiveness to cAMP. Therefore, SF-1 is critical to the mouse GnRHR gene promoter, activating the PKA pathway. Also, SF-1 is able to interact with various transcription factors such as GATA and LIM related factors (Pincas et al., 2001), AP-1 and GRAS (Duval et al., 1997a), Sp1 (Liu and Simpson, 1997), C/EBPβ (Reinhart et al., 1999), SOX9 (De Santa Barbara et al., 1998), and Wilms' Tumor 1 (Nachtigal et al., 1998).

Activating Protein-1. The transcription factor, AP-1, also known as a TPA-response element (TRE), is mainly comprised of Jun and Fos protein dimers. Activating protein-1 controls gene regulation in response to stimuli of cytokines, growth factors, stress signals and bacterial and viral infections (Hess et al., 2004). These proteins contain a basic DNA-binding domain with a leucine zipper region. The leucine zipper controls dimerization such as the specificity and stability of homo- and heterodimers that comprise the AP-1 complex (Wagner, 2001; Eferl and Wagner, 2003). Jun proteins can

form either homo- or heterodimers, whereas Fos proteins bind as a heterodimer with Jun proteins to increase DNA-binding stability (Hess et al., 2004). In addition, Jun proteins can form heterodimers with many other transcription factors to bind AP-1 elements.

Cheng et al. (2000b) demonstrated that an AP-1 site located in the human GnRHR promoter is responsible for the down-regulation of the GnRHR gene following treatment with GnRH. Although mutation of the AP-1 site abolished down-regulation by GnRH, it had no effect on basal promoter activity. An up-regulation of c-Fos and c-Jun proteins following treatment of αT3-1 cells with a GnRH agonist indicated that AP-1 plays a critical role in the regulation of GnRH binding to its receptor (Cheng et al., 2000b). In addition, the human GnRHR gene promoter contains 2 putative AP-1/cAMP response element binding (CREB) sites; human GnRHR (hGR)-AP/CRE-1 and hGR-AP/CRE-2 that are involved in cAMP responsiveness of αT3-1 cells (Cheng and Leung, 2001). Mutations of these putative elements decreased forskolin-stimulated promoter activity. Conversely, there are other transcription factors involved in cAMP responsiveness in addition to AP-1 because these mutations did not completely abolish the cAMP stimulatory effect (Cheng and Leung, 2001).

In addition to GnRH responsiveness of the GnRHR promoter, an AP-1 binding site in the mouse GnRHR gene promoter is also important in basal expression (Duval et al., 1997a), binding fos/jun family members (White et al., 1999). The mouse GnRHR promoter is mediated by GnRH and activin via a multifactor ADP-ribosylation factor (ARF) protein complex, including AP-1 (Fos/Jun) and SMAD proteins (Norwitz et al., 1999). Ellsworth and associates (2003b) suggested that the AP-1 element which binds

JunD, FosB and c-Fos residing within -600 bp of 5' flanking region in the mouse GnRHR gene promoter requires JNK activation upon stimulation by GnRH. Also, activin responsiveness of the mouse GnRHR gene promoter is conferred by an AP-1 element residing upstream within the GRAS element (Ellsworth et al., 2003a).

Similar to GnRH, estrogen is one of the most critical regulators of GnRHR gene expression. Ovarian cancer (OVCAR-3) and breast cancer (MCF-7) cells treated with E_2 caused repression of the human GnRHR promoter via estrogen receptor (ER) α and AP-1 elements. Although, c-Jun and c-Fos bound to the AP-1 element, their DNA binding activity was not affected by E_2 treatment. Thus, E_2 can regulate expression of the GnRHR gene through an ERα-dependent mechanism that utilizes an AP-1 element. The E_2 repression of the GnRHR promoter is also regulated by an indirect mechanism involving CBP and other potential transcription factors (Cheng et al., 2003).

GATA Factors. Two copies of a highly conserved, zinc finger DNA binding domain are characteristic of the GATA binding protein family (Steger et al., 1994). The GATA factors are comprised of a C-terminal zinc finger and basic domain that is conserved within the GATA protein family (Lowry and Atchley, 2000) and each GATA member recognizes a specific DNA-binding site, (A/T)GATA(A/G) (Lawson et al., 1996). Proteins in the GATA family play important roles in various areas of development and are expressed in many different tissues. They are involved in cell differentiation, organ morphogenesis, and tissue-specific gene expression. These proteins

are expressed in the placenta, pituitary, gonads, brain, heart, hemopoietic system and gut (Tremblay and Viger, 2001; La Voie, 2003).

There are currently 2 classes of GATA proteins; the hematopoietic (GATA-1/2/3) and cardiac (GATA-4/5/6) groups (Tremblay and Viger, 2001; La Voie, 2003). GATA-1 is involved in the development of erythropoietic stem cells (Evans and Felsenfeld, 1989; Steger et al., 1994; Lawson et al., 1996), whereas GATA-2 is expressed in embryonic and adult tissues (Yamamoto et al., 1990; Steger et al., 1994; Lawson et al., 1996). GATA-3 is highly expressed in developing neural tissue, placenta, pituitary, T cells, and a wide variety of other tissues (Yamamoto et al., 1990; Steger et al., 1994; Lawson et al., 1996). The fourth member, GATA-4, is mainly involved in developing cardiac tissue and is detected in primitive endoderm, intestinal epithelium, and T cells. It is also expressed in reproductive tissues such as the gonads and pituitary-derived cells. (Arceci et. al., 1993; Kelley et al., 1993; Steger et al., 1994; Lawson et al., 1996). The most recently discovered GATA factors, GATA-5 and -6 were originally found in chickens and are expressed in the gonadal, gut, and heart tissue (Laverriere et al., 1994; Lawson et al., 1996).

The GATA factors are critical in mammalian reproductive development and function. Reproductive gene expression is regulated in the hypothalamus and anterior pituitary gland by GATA family members (La Voie, 2003). Specifically, GATA-4 and/or GATA-6 has been found in the granulosa layer of healthy follicles and is involved in the transcription of gonadotropin-regulated genes including aromatase (Tremblay and Viger, 2001; 2003), steroidogenic acute regulatory protein (StAR), and inhibin (Gillio-

Meina et al., 2003). In addition, GATA-4 expression has also been found in a majority of germ cells prior to puberty in humans. It has also been implicated in steroid hormone production by gonadal cells (La Voie, 2003). Several members of the GATA transcription factor family regulate GnRH gene transcription through binding sites within the GnRH neuron-specific enhancer in the GnRH-secreting hypothalamic neuronal cell line, GT1-7. GATA-4 was detected in an enhancer specific binding complex suggesting its importance in regulating neuron-specific expression of the GnRH gene in hypothalamic neurons (Lawson et al., 1996). In summary, the GATA family of transcription factors is important in the reproductive axis, impacting the expression and function of the hypothalamus, pituitary and gonads.

Nuclear Factor-κB. Nuclear factor-κB plays an important role in the immune system, regulating the expression of cytokines, growth factors, and effector enzymes in response to the activation of receptors involved in the immune systems (Silverman and Maniatis, 2001; Hayden and Ghosh, 2004). Recent studies indicated that NF-κB is involved in systems other than the immune system. For example, it is involved in embryonic development, the development and physiology of tissues (mammary gland, bone, and skin) and the central nervous system (Hayden and Ghosh, 2004). The mammalian NF-κB family consists of 5 members; p65 (RelA), RelB, c-Rel, p50 (NF-κB1) and p52 (NF-κB2). They may exist as either homodimers or heterodimers bound to IκB family proteins in their inactive state (Siebenlist et al., 1994; Baeuerle and Henkel, 1994; Hayden and Ghosh, 2004). The most common form of NF-κB exists as a

heterodimer consisting of the p50 and p65 subunits (Hong et al., 2003). As NF-κB binds to IκB proteins, it forms a NF-κB:IκB complex, preventing the complex from translocating to the nucleus and maintaining NF-κB in an inactive state. Phosphorylation and degradation of IκB proteins allows NF-κB dimers to translocate into the nucleus, where it binds to specific sequences in the promoter or enhancer regions of target genes (Baldwin, 1996; Hayden and Ghosh, 2004).

Nuclear factor-κB is involved in a variety of biological processes and many different cell types including the anterior pituitary gland. Therefore, NF-κB may play an important role in gene regulation involved in the anterior pituitary gland, suggesting an important role in reproductive functions. Nuclear factor-κB is involved in the stimulation of oxidative stress in the pituitary corticotrope cell line, AtT20 (Karalis et al., 2004). Activation of NF-κB complexes via the interaction between interleukin-1 (IL-1) and two different receptors, IL-1 receptor type I and type II, was detected in somatotrope cells (Parnet et al., 2003). Delfino and Walker (1998) suggested NF-κB proteins may also play a role in stage-specific gene expression during spermatogenesis. Mullerian inhibiting substance (MIS) expression in Sertoli cell cultures is regulated by the interaction between SF-1 and NF-κB. Steroidogenic factor-1 is stimulated by its interaction with NF-κB through the p50 subunit; however, SF-1 activation is inhibited by the p65 subunit of NF-κB. Therefore, different subunits of NF-κB play divergent roles in the mechanisms underlying MIS expression by meiotic germ cells in the testes (Hong et al., 2003).

Specificity Protein Family. The specificity protein family is comprised of 9 members; Sp1-9 (Suske, 1999; Suske et al., 2005; Sahara et al., 2007). Each member of the Sp family consists of 3 zinc finger DNA binding domains located at the C-terminus, in addition to various serine/threonine domains at the N-terminal region (Wooten and Ogretmen, 2005). The Krüppel-like factor (KLF) proteins represents a subgroup of a larger class of transcription factors (Lomberk and Urrutia, 2005). Similar to members of the Sp family, these factors bind to GC-GT rich sites and CACC boxes of many different gene promoters (Sogawa et al., 1993; Crossley et al., 1996; Shields and Yang, 1998; Lomberk and Urrutia, 2005). They also share a highly conserved DNA-binding domain, the most common transcription factor motif within the human genome. This binding domain consists of 3 Cys^2/His^2 zinc fingers (Philipsen and Suske, 1999; Bieker, 2001). Krüppel-like transcription factors can act as either activators or repressors depending on promoter specificity. In order to regulate transcription of their target genes, they can also interact with co-activators and co-repressors via different mechanisms (Lomberk and Urrutia, 2005).

The various specificity proteins play divergent roles throughout many tissues. Specificity protein 4 is mainly found in neuronal tissues and abundant in epithelial tissues, developing teeth and testes (Philipsen and Suske, 1999), whereas Sp1, Sp2 and Sp3 are expressed ubiquitously (Wooten and Ogretmen, 2005). Specificity protein 1, 3 and 4 contain similar sequences, binding patterns and structure. However, the Sp family members have some differences among one another. For example, Sp1 contains an inhibitory domain at the N-terminus, whereas Sp4 has an inhibitory domain located near

the zinc fingers. Specificity proteins 1 and 3 both have high affinities for GC-rich sequences and are 90% homologous within their zinc finger domains. On the contrary, Sp2 has a high affinity to bind to GT-rich sequences and has the least homology to the other Sp family members (Wooten and Ogretmen, 2005). In addition to activating transcription, Sp3 can also act as a repressor (Al-Sarraji et al., 2005; Wooten and Ogretmen, 2005). Specificity proteins 5 and 8 have zinc finger domains that are 93.8% similar to one another. Specificity protein 8 may also bind to Sp1 binding sites due to its similarity to the Sp1 zinc finger domain. During central nervous system development, the expression pattern of Sp5 and Sp8 was similar within the mouse (Treichel et al., 2003). Specificity protein 5 can act as a transcriptional repressor, containing 3 repressor domains. This Sp family member also represses Sp1-regulated target genes and has a similar binding specificity as Sp1 (Fujimura et al., 2007). During limb development, Sp8 acts as a transcriptional activator. However, it also has the capability to act as a transcriptional repressor because it has several mSin3a core consensus sequences and polyalanine tracts (Kawakami et al., 2004). The 3 transcription factors, Sp5, 8 and 9 are expressed in the embryonic mouse forebrain in unique patterns and are also associated with forebrain signaling centers (Sahara et al., 2007).

Functional Sp1 and Sp3 are present in many different mammalian gene promoters including: monocyte chemoattractant protein 1 (MCP-1; Ping et al., 2000), tissue factor (Oeth et al., 1997), rat luteinizing hormone-β (LH-β; Kaiser et al., 2000), bovine CYP11A (Liu and Simpson, 1997) and the long terminal repeat of the HIV-1 promoter (Majello et al., 1994). Interestingly, NF-κB interacts with both Sp1 and Sp3 to regulate

transcriptional activity of various mammalian gene promoters (Majello et al., 1994; Oeth et al., 1997; Ping et al., 2000). In addition, Sp1 can regulate the LH-β gene promoter in response to GnRH suggesting a role for Sp1 in transcriptional regulation in gonadotrope cells (Kaiser et al., 1998; 2000). Therefore, the Sp family of transcription factors can play a critical role in the transcriptional regulation of gonadotropic genes.

Progesterone Receptor. Progesterone receptor is expressed in many tissues and consists of 2 isoforms; PR-A and PR-B. Progesterone receptor-A is a truncated form of PR-B as PR-B contains an additional 164 amino acids at the N-terminus (Schott et al., 1991; Kraus et al., 1993). The transcriptional activity of PR-A is both cell- and gene-dependent, whereas PR-B acts as a stronger transcriptional activator (Cheng et al., 2001a). In addition, there is a variation in the ratio of PR-A:PR-B isoforms within target tissues indicating that cell-specific responses are due to the differential expression of these isoforms (Shyamala et al., 1998; Mulac-Jericevic and Conneely, 2004).

However, little is known about the role of the PR isoforms in the pituitary gland. Turgeon and Waring (2006) determined PR-A and PR-B mRNA levels in rat and mouse cultured pituitary cells as well as LβT2 cells. It was concluded that PR-A was the most abundant isoform in both cell lines. They also established roles for E_2 and P_4 in PR isoform expression within these cell lines. Total PR mRNA within rat pituitaries is up-regulated by E_2 (Turgeon et al., 1999; Sanchez-Criado et al., 2004). Also, rat and mouse pituitary cultures responded to E_2 stimulation with increased expression of both PR isoforms, whereas PR-A and -B mRNA levels did not respond to E_2 in LβT2 cells

(Turgeon and Waring, 2006). Additionally, the PR-B isoform has a more robust response to E_2 in rat than mouse pituitary cultures, decreasing the PR-A:PR-B isoform ratio (Turgeon and Waring, 2006). Therefore, regulation of PR isoforms by E_2 in gonadotropes contributes to LH secretion differences between the rat and mouse. Luteinizing hormone secretion is regulated by either GnRH self-priming or P_4 augmentation, which are both estrogen and transcription-dependent events (Turgeon and Waring, 2006).

Transcriptional regulation of human GnRHR gene expression by P_4 is different at the pituitary and placental levels (Cheng et al., 2001a). For instance, human GnRHR promoter activity was decreased in both pituitary and placental cells following overexpression of PR-A. However, overexpression of PR-B in the presence of the human GnRHR promoter demonstrated cell-dependent transcriptional activity because it functioned as a repressor in the pituitary and an activator in the placenta (Cheng et al., 2001a). An et al. (2005) also indicated differential roles for the PR isoforms in regulating the human GnRHR promoter in TE-671 (human neuronal medullablastoma) cells. Progesterone receptor-A acted as a transcriptional repressor, whereas PR-B functioned as an activator following treatment with P_4. Thus, PR-A can act on the GnRHR promoter in a gene-specific manner and PR-B could be a transdominant regulator of PR-A (An et al., 2005). Additionally, these results indicate that PR-A is more dominant compared to PR-B. Overall, P_4 may regulate the GnRH system by different pathways utilizing the 2 different PR isoforms (An et al., 2005).

Gonadotropin-Releasing Hormone Receptor in Swine

In addition to its physiological importance, the porcine GnRHR gene is located on porcine chromosome 8, in close proximity to a quantitative trait locus (QTL) for ovulation rate (Rohrer et al., 1999). Thus, the GnRHR gene is both a physiological and positional candidate for genes influencing reproductive traits, such as ovulation rate (Jiang et al., 2001; Campbell et al., 2003b). In addition to the QTL for ovulation rate on chromosome 8, other QTLs have also been identified for prenatal survival, teat number, litter size and uterine capacity (Rohrer et al., 1999; King et al., 2003). Therefore, the porcine GnRHR gene may represent a candidate gene for these traits as well. The identification of additional QTLs associated with reproductive traits and correlation of these markers with physiological mechanisms could dramatically improve breeding programs within the swine industry.

Genomic sequencing of the porcine GnRHR gene revealed differences in promoter sequences between the Chinese Meishan and White Crossbred lines of pigs (Jiang et al., 2001). In addition, our laboratory has identified additional bp alterations between these lines that result in functional differences in GnRHR promoter activity (McDonald, 2005). The Chinese Meishan is a very prolific breed farrowing about 4 to 5 more piglets per litter compared to occidental breeds, largely due to increased ovulation rate. Furthermore, GnRHR mRNA levels were higher in ovariectomized Meishan gilts compared to ovariectomized white crossbred gilts (Bass, 2005). Therefore, this breed represents an excellent model to study the porcine GnRHR gene and its effects on

ovulation rate, a major contributor to litter size (Christenson et al., 1993; White et al., 1993; King et al., 2003).

Genes Influencing Prolificacy

Sheep

The Fec^B (Booroola) genotype is associated with the physiological and genetic characteristics of the Booroola Merino, one of the most prolific breeds of sheep in the world (Bindon, 1984) as well as Garole and Javanese sheep (Souza et al., 2001; Wilson et al., 2001; Mulsant et al., 2001; Davis et al., 2002). The Fec^B genotype has been associated with increased ovulation rate and litter size, due to a single natural mutation (Bindon, 1984; Campbell et al., 2003a). Ewes with the Fec^B genotype ovulated more small follicles compared to wild type ewes. Although there was a difference in ovulation rate between ewes carrying the Fec^B genotype and wild type ewes, they both had identical gonadotropic hormonal concentrations (Campbell et al., 2003a). Therefore, rather than enhanced FSH and LH levels, the increased ovulation rate is likely due to mechanisms that alter responsiveness of the ovary to gonadotropic stimulation as a result of the Fec^B genotype (Campbell et al., 2003a).

Further studies indicated that sheep with the Fec^B genotype contained a natural mutation in the highly conserved intracellular serine threonine kinase signaling domain within the bone morphogenetic protein receptor 1B (BMPR-1B) gene located on chromosome 6 (Souza et al., 2001; Wilson et al., 2001). Bone morphogenetic proteins (BMPs) such as BMP-2, -4 and -6 bind to BMPR-1B through common pathways and are

members of the transforming growth factor-β (TGF-β) superfamily (Campbell et al., 2006). Bone morphogenetic proteins are multifunctional proteins that regulate growth and differentiation in various cell types (Wilson et al., 2001). Expression of BMPR-1B was detected in oocytes and granulosa cells within the ewe ovary, suggesting an important role for BMPR-1B in development of these cell types (Wilson et al., 2001). The BMPRs present within the ewe ovary may be involved in differentiation of granulosa cells by stimulating FSH action (Souza et al., 2002). A recent study by Campbell et al. (2006) indicated a major function of BMPs in controlling ovarian somatic cell function. Increased differentiation of granulosa and theca cells following stimulation with BMPs, gonadotropins and insulin growth factor-1 (IGF-1) were detected in ovarian tissue from animals with the Fec^B genotype compared to controls. Overall, the Fec^B genotype causes the maturation of ovarian follicles and disrupts the normal follicular mechanism for selection in ewes (Campbell et al., 2006).

An additional genotype that influences prolificacy in sheep is $FecX^1$ (Inverdale; Davis et al., 2001). Sheep with the $FecX^1$ genotype contain a mutation in the BMP15 gene, another member of the TGF-β superfamily that has been mapped to the X chromosome (Galloway et al., 2000). The BMP15 gene is expressed in oocytes, representing an important gene for female fertility. However, natural mutations in the BMP15 gene can cause an increase in either ovulation rate or infertility phenotypes (Galloway et al., 2000). Heterozygous genotypes for the BMP15 gene ($FecX^1/FecX^+$) were associated with increased ovulation rates and multiple births (Davis et al., 1991), whereas homozygous genotypes ($FecX^1/FecX^1$) were associated with primary ovarian

failure (Davis et al., 1992). The BMP15 gene influenced fecundity in the Inverdale (Davis et al., 1991), Hanna (Davis et al., 1991), Belclare (Hanrahan et al., 2004), Cambridge (Hanrahan et al., 2004), and Lacaune (Bodin et al., 2002) breeds of sheep.

Investigators examined the BMPR-1B and BMP15 genes in Small Tailed Han sheep, a prolific breed in China, to determine their association with prolificacy (Chu et al., 2007). This breed of sheep contains mutations in both the BMPR-1B and BMP15 genes. Extensive analysis of these 2 genes in the Small Tailed Han indicated that ewes carrying both BMPR-1B and BMP15 mutations had greater litter sizes compared to ewes harboring either single mutation. Therefore, using both BMPR-1B and BMP15 genes for marker-assisted selection will help increase litter size in sheep and contribute to an increase in economic value to sheep producers (Chu et al., 2007).

Mice

Ovulation rate is partially regulated by activity of gonadotropins in circulation and ovarian sensitivity to these hormones (Land and Falconer, 1969). To examine sensitivity of the ovary to gonadotropins among mouse strains, a line selected for increased litter size and reduced body size was compared to unselected mice after administration of FSH followed by hCG. The number of oocytes ovulated were used to measure ovarian sensitivity to these hormones (Durrant et al., 1980). Gonadotropin treatment resulted in increased ovulation rate and decreased early embryonic death for selected compared to unselected mice, indicating differences in ovarian sensitivity to gonadotropins between lines (Durant et al., 1980). Pomp and associates (1988) suggested

that selection of mice for large litter size resulted in increased ovulation rates during subsequent generations due to either a decrease in LH receptors or factors related to growth. In order to understand how increased litter size and mature body weight affected reproduction, Kirkpatrick et al. (1998b) identified QTLs for prolificacy and growth in mice. They found 4 chromosomal regions that were associated with litter size. However, 2 of these regions associated with litter size were also related to a growth QTL, potentially impacting the positive genetic relationship among these traits (Kirkpatrick et al., 1998b).

Pre-implantation embryonic development has also been used as a selection marker for litter size in mice (Al-Shorepy et al., 1992). An allele of the *ped* gene has been correlated with increased cleavage rates during embryonic development. The increased rate of cleavage associated with the *ped* gene could result in larger litter sizes. Therefore, alleles of the *ped* gene, or loci in close proximity to the *ped* gene, may be one of the genes involved in prolificacy of mice (Al-Shorepy et al., 1992). Another gene that could potentially impact prolificacy in mice is the inhibin α-subunit gene. Cho et al. (2001) constructed a transgenic mouse that harbored the rat inhibin α-subunit gene. Overexpression of the rat inhibin α-subunit gene led to a disruption in the inhibin:activin ratio, causing various reproductive defects including reduced ovulation rates and fertility. Since, inhibin and activin are important for normal gametogenesis and fertility, the inhibin α-subunit gene is a likely candidate gene for prolificacy in mice (Cho et al., 2001). Overall, mice have many potential QTLs for prolificacy, however many gene associations remain to be determined.

Swine

Females from the Chinese Meishan breed are very prolific, producing approximately 4 to 5 more piglets per litter compared to occidental breeds (Haley and Lee, 1993). Therefore, the Chinese Meishan breed has been widely studied to determine potential genes that impact prolificacy. Since estrogen and its receptor play an important role in the reproductive function in mammals, Rothschild and associates (1996) investigated whether the ER gene has an impact on litter size in pigs. A specific allele of the ER gene, initially found in the Meishan, was associated with increased litter size. However, a beneficial ER allele was associated with increased litter size at first parity and the average of all parties in the Large White breed (Rothschild et al., 1996). Short et al. (1997) investigated 4 white-crossbred, commercial swine lines to determine associations between the ER gene and growth and reproductive traits. These scientists identified a specific allele for the ER gene that was correlated with increased litter size and number of piglets born alive (Short et al., 1997).

In addition to the ER gene, a polymorphism in the prolactin receptor (PRLR) gene may also be associated with increased litter size (van Rens et al., 2003). However, it is still unknown whether the polymorphism causes increased litter size or if it is merely a marker for a linked gene. For example, a 50% Landrace/50% Meishan crossbred line indicated that the PRLR gene, or a closely linked gene, was associated with reproductive traits such as ovarian weight, uterine capacity, ovulation rate and placental weight. Therefore, the changes in these traits may eventually lead to differences in litter size (van Rens et al., 2003).

CHAPTER III

MATERIALS AND METHODS

Lines of Swine

The gonadotropin releasing hormone receptor (GnRHR) gene promoter from 3 lines of swine with divergent ovulation rates were utilized in these studies. A control white-crossbred line (Control) was used that displays reproductive traits common to sows used in commercial hog production. The Index line was developed at the University of Nebraska-Lincoln (UNL) by Dr. Rodger Johnson and was selected based on an index of ovulation rate and embryonic survival at 50 days of gestation (Johnson et al., 1984). After more than 14 generations of selection, females from this line ovulated approximately 7 more oocytes per estrous cycle, resulting in 1.4 more piglets per litter compared to the Control line (Johnson et al., 1999). The Chinese Meishan is a highly prolific breed, producing approximately 4 to 5 more piglets per litter compared to occidental breeds, largely due to increased ovulation rate (Christenson et. al, 1993; White et al., 1993). Therefore, the Chinese Meishan represents a unique model to study genes that influence ovulation rate, such as the GnRHR gene promoter.

GnRHR Gene Promoter Isolation

Our laboratory isolated the porcine GnRHR gene promoter by inverse PCR using primers specific for the 1154 bp of 5' untranslated region previously reported by Jiang and associates (2001; GenBank Accession No. AF227685). Genomic DNA preparations

were made by a partial restriction digest using *Sau*3AI or *Eco*RI. Fragments were self-ligated using a high concentration of T4 DNA ligase (4 U/µl, New England Biolabs, Beverly, MA) and a low concentration of DNA (3 µg/µl). Subsequently, PCR was performed on the circularized pieces of DNA, with primers selected from the known flanking sequence. The PCR primers that were chosen were in reverse orientation compared to traditional PCR primers. Polymerase chain reaction products were subcloned and sequenced at the UNL Genomics Core Research Facility. Sequencing data representing 5197 bp of 5' flanking region for the porcine GnRHR gene is available in GenBank (Accession No. AY166667; Cederberg et al., unpublished data). Based on this sequence, Index and Meishan full-length promoters were isolated from genomic DNA using primers specific for the Control promoter. Subsequently, the Index and Meishan promoters were also sequenced.

Plasmid Preparation

Reporter Vectors. The full-length Control GnRHR gene promoter was sub-cloned into the pGL3 basic vector (Figure 3.1; Promega Corp., Madison, WI) using *SacI* and *SmaI* restriction endonuclease sites. Therefore, the test vector consisted of the full-length GnRHR gene promoter fused to the cDNA encoding luciferase. The Index and Meishan full-length promoters were sub-cloned into luciferase reporter vectors as described above, utilizing *SacI* and *XhoI* endonuclease sites and *SacI* and *NheI* sites, respectively. The Rous Sarcoma Virus promoter fused to the cDNA encoding β-

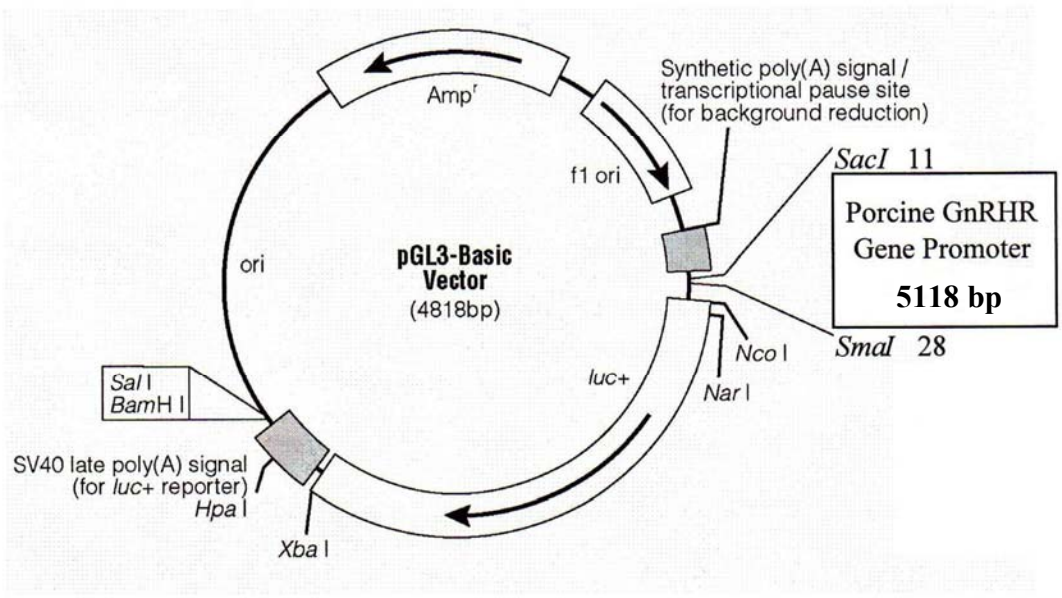


Figure 3.1. The full-length porcine GnRHR gene promoter was sub-cloned into the pGL3-basic vector by *Sac*I and *Sma*I restriction endonuclease recognition sequences.

galactosidase (RSV- βgal, Stratagene, La Jolla, CA.) was used as a control to normalize the transfections.

Block Replacement Mutations. Primers were designed to replace a specific element within the GnRHR gene promoter with a restriction enzyme site. Polymerase chain reaction was used to replicate both the sense and anti-sense strands of the DNA using specific primers (Table 3.1; Integrated DNA Technologies, Coralville, IA) for the region to be mutated. The PCR reaction consisted of 10X AccuPrime *Pfx* reaction mix (buffer, 1 mM $MgSO_4$ and 0.3 mM dNTPs; Invitrogen Life Technologies Corp., Carlsbad, CA), 10 μM primers, 50 ng/μl DNA template, AccuPrime *Pfx* DNA polymerase (Invitrogen Life Technologies Corp.) and Millipore water to bring the final volume to 50 μl. The PCR cycling conditions to generate the mutation in each strand were as follows: Step 1 - 94°C for 2 min; Step 2 - 94°C for 15 sec; Step 3 - 55°C for 30 sec; Step 4 - 68°C for 2 min; Step 5 - repeat step 2 through 4, 35 times; and Step 6 - 68°C for 10 min.

The sequences that were generated from the above PCR reaction were utilized in a second PCR reaction to design the template DNA. The second PCR reaction used the same PCR cycling conditions as previously described. After completion of the second PCR reaction, the products were purified using the QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA). To replace specific transcription factor binding sites, block replacement mutation vectors contained: a *PstI* restriction endonuclease site in place of the GATA element at -845/-840 bp (Mμ845GATA-4pGL3) or a *Bcu/SpeI* site substituted

for the GATA element at -1694/-1689 bp (Mμ1690GATA-4pGL3) within the Meishan promoter; an *EcoRI* site replacing the NF-κB element at -1699/-1694 bp (C/Iμ1699NF-κBpGL3) or an *EcoRI* site in place of the NF-κB binding site at -1689/-1684 bp (C/Iμ1689NF-κBpGL3) specific to the Control/Index promoter; and a *NotI* (μSF-1pGL3) or *SacI* (μOct-1pGL3) site in exchange for the SF-1 (-1760/-1753) or Oct-1 (-1731/-1724) binding sites, respectively, within homologous portions of the promoter from all three swine lines.

The PCR reaction was purified with the QIAquick PCR Purification Kit (Qiagen, Inc., Valencia, CA). Five volumes of buffer PB1 was added to one volume of the PCR sample, mixed and placed in a QIAquick spin column within the 2-ml collection tube that was provided. Next, the sample was centrifuged using a Biofuge Pico microcentrifuge (Kendro Lab Products, Hanau, Germany) at 16,000 x *g* for 1 min. The flow-through was discarded and the spin column was placed back into the same collection tube. Next, the column was washed with 750 μl of buffer PE and centrifuged at 16,000 x *g* for 1 min. The flow-through was discarded and the spin column was placed back in the same tube and centrifuged for 1 min. The QIAquick column was placed in a clean 1.5-ml microcentrifuge tube and DNA was eluted from the QIAquick column upon addition of 50 μl of buffer EB and centrifugation at 16,000 x *g* for 1 min. After PCR purification, vectors containing the full-length GnRHR gene promoter and PCR products were double digested with restriction endonucleases to ligate the PCR product (insert) into the digested vector containing the full-length GnRHR gene promoter (template). Following the double restriction digest, the insert and template were gel extracted and quantitated.

To set up ligations, insert to template ratios were 1:0 (control), 1:1, 1:5 or 1:10. The ligation reaction containing T4 ligase (5U/µl, Fermentas, Glen Burnie, MD) and T4 ligase buffer (Fermentas) was incubated overnight at 15°C.

Gel Extraction. Plasmid fragments were extracted from agarose gels with the Perfectprep® Gel Cleanup Kit (Eppendorf, Westbury, NY). The excised gel slice was weighed with a maximum of 400 mg in a 2-ml microcentrifuge tube. Next, 3 volumes of binding buffer were added for every volume of each gel slice (1 mg of weight equaled 1 µl of volume). Next, gel slices were incubated at 50°C for 5 to 10 min in a heat block and vortexed every 2 to 3 min. After the gel slice was completely dissolved, an equal volume of isopropanol was added to the original gel slice volume and mixed by inversion. The sample (up to 800 µl) was placed in a spin column in a 2-ml collection tube and centrifuged using a Biofuge Pico (Kendro Lab Products) at 6,000 to 10,000 x *g* for 1 min. Next, the filtrate was discarded and the spin column was replaced in the same collection tube. If the sample was larger than 800 µl the sample was reloaded and centrifuged again. Diluted wash buffer was prepared by adding 600 µl of 100% ethanol to 150 µl of concentrated wash buffer. Next, 750 µl of diluted wash buffer was added to each spin column and columns were centrifuged for 1 min at 6,000 to 10,000 x *g*. The filtrate was discarded and the spin column was replaced in the same collection tube. The spin column in the collection tube was centrifuged for an additional min at 6,000 to 10,000 x *g* to remove any residual wash buffer. The spin column was then placed in a new 2-ml collection tube, 30 µl of elution buffer was added to the center of the spin

column and it was centrifuged for 1 min at 6,000 to 10,000 x *g*. The plasmid fragments were quantitated with a Pharmacia GeneQuant spectrophotometer (Pfizer, New York, NY) using A_{260} and A_{280} values and allowed to ligate overnight at 15 °C.

Transformation. Plasmids were transformed utilizing Rb-Cl competent DH5α cells (Invitrogen Life Technologies Corp.). A total of 5 µl of each plasmid ligation reaction was added to 50 µl of DH5α cells, gently mixed and incubated on ice for a total of 30 min. The reaction was then heat shocked for 30 sec at 42°C on a heat block and incubated on ice for 2 min. Next, 200 µl of SOB media (2% tryptone, 0.5% yeast extract, 8.55 mM sodium chloride, 250 mM potassium chloride, 2 M magnesium chloride) was added to each reaction and placed in a 37°C shaking incubator for 1 h. Plasmids were plated (50-200 µl) on LB agar plates (Sigma Chemical Co., St. Louis, MO) containing ampicillin and incubated inverted at 37°C overnight. Approximately 16 h later, colonies were counted, evaluated, stored at 4°C for up to 1 mo and utilized in alkaline lysis mini plasmid preparations.

Alkaline Lysis Mini Plasmid Preparation. Plasmid DNA was extracted from Rb-Cl competent DH5α cells (Invitrogen Life Technologies Corp.) for screening purposes. A single colony containing an ampicillin resistant plasmid in Rb-Cl cells was inoculated overnight and grown in 2 ml of LB media (Sigma Chemical Co.) containing 0.05 mg/ml ampicillin shaking at 37°C. Cells were pelleted by centrifuging 1 ml of culture at 16,000 x *g* for 1 min with a Biofuge Pico microcentrifuge (Kendro Lab

Products). The supernatant was removed and the cell pellet was resuspended in 100 µl GTE [50 mM glucose, 25 mM Tris-Cl, 10 mM ethylenediaminetetraacetic acid (EDTA)] and incubated at room temperature for 5 min. Next, 200 µl of NaOH/SDS (0.2 N NaOH, 1% (wt/vol) sodium dodecyl sulfate) was added to each sample, mixed and incubated on ice for 5 min. Following the incubation, 150 µl of 5 M potassium acetate (29.5 ml glacial acetic acid, potassium hydroxide (KOH) pellets, pH 4.8) was added to each sample, vortexed for 2 s, and incubated on ice for an additional 5 min. The samples were centrifuged at 16,000 x *g* for 3 min and the supernatant was placed in a clean 1.5-ml microcentrifuge tube. The supernatant was mixed with 800 µl of 100% ethanol and incubated at room temperature for 2 min. The samples were then centrifuged at 16,000 x *g* for 1 min to pellet plasmid DNA and RNA. The supernatant was then removed and the pellet was washed with 1 ml of 70% ethanol. The samples were centrifuged for an additional min at 16,000 x *g*, the supernatant was removed again, and the pellet dried at room temperature. Once dried, the pellet was resuspended in 1X tris-ethylenediaminetetraacetic acid (TE, pH 8.0). Following resuspension, 1 µl of RNase A was added to each sample and incubated at 37°C for 30 min. Plasmids were later screened by restriction digests for confirmation prior to being utilized in a support protocol.

Midi Plasmid Purification. Plasmids were prepared for transfection using a Plasmid Purification Midi Kit (Qiagen, Inc.) and confirmed with restriction enzyme digests. An overnight culture, shaking at 37°C, was inoculated with 100 ml of LB broth

containing 0.05 mg/ml ampicillin and a bacterial stock containing the vector of interest. The bacterial cells were harvested by centrifugation at 6,000 x *g* for 15 min at 4°C using a Sorvall RC2-B centrifuge (Du Pont Co., Newton, CT). The bacterial pellet was resuspended in 4 ml of buffer P1 and completely vortexed until no cell clumps remained. Next, 4 ml of lysis buffer P2 was added followed by a 5 min incubation at room temperature. Then, 4 ml of pre-chilled precipitation buffer P3 was added to each sample and incubated on ice for 15 min. Following incubation on ice, samples were centrifuged at 20,000 x *g* for 30 min at 4°C using a Sorvall RC2-B centrifuge (Du Pont Co.).

Each resin column was equilibrated prior to the addition of sample (cell lysate) with 4 ml buffer QBT. After each sample was added to the columns, they were washed twice with 10 ml of buffer QC. Next, the plasmid DNA was eluted upon the addition of 5 ml of buffer QF to each column. The plasmid DNA was precipitated by the addition of 3.5 ml of room temperature isopropanol and centrifuged at 15,000 x *g* for 30 min at 4°C using a Sorvall RC2-B centrifuge (Du Pont Co.) The supernatant was decanted and pellets were washed with 2 ml of room temperature 70% ethanol and centrifuged at 15,000 x *g* for 10 min at 4°C using a Sorvall RC2-B centrifuge (Du Pont Co.). Next, the pellets were air dried for 5-10 min and resuspended in approximately 250 µl of Millipore water. The purified plasmids were quantitated utilizing a Lambda EZ 150 spectrophotometer (Perkin Elmer, Boston, MA) and screened using restriction enzymes. To confirm that vectors contained the insert, they were sequenced prior to use in transient transfection assays. Finally, vectors were stored at -20°C until use.

Cell Culture

αT3-1 cells (Dr. Pam Mellon, Salk Institute, La Jolla, CA) were maintained on 150-mm cell culture plates (Corning Inc., Corning, NY) in 20 ml of αT3-1 media. The media was comprised of high glucose Dulbecco's Modified Eagle Medium (DMEM; Mediatech Inc., Herndon, VA) with 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 5% horse serum and 5% fetal bovine serum (Gibco, Grand Island, NY). The cultures of the αT3-1 cells were maintained at 37°C in a humidified 5% CO_2 in air environment.

Transient Transfections

Day 1. At approximately 85% confluency**,** αT3-1 cells were trypsinized, counted, and plated for transfection utilizing a liposome-mediated protocol. The media was aspirated from the cells and they were washed with 10 ml of 1X phosphate-buffered saline (PBS) 2 times. Cells were trypsinized by adding 3 ml of 1X trypsin-EDTA (Mediatech Inc.) and incubated for 5 min at 37°C in a humidified 5% CO_2 in air environment. After the cells detached from the plate, 7 ml of αT3-1 media was added to disperse cell clumps and cells were transferred to a 50-ml conical tube. A 100 µl sample of cells was diluted in 900 µl of 1X PBS and 10 µl of the dilution was loaded onto a hemacytometer to perform cell counts. Approximately 2 x 10^6 αT3-1 cells were plated in a 6-well culture plate (Corning Inc., Corning, NY) to achieve 50% confluency at transfection. Each well contained 2 ml of αT3-1 media.

Day 2. A 1.5-ml microcentrifuge tube for each test vector contained 291 µl of serum-free DMEM and 9 µl of Fugene6 (Roche Diagnostics Corp., Indianapolis, IN). Each test vector was transfected in triplicate and 0.9 µg/well of test vector and 0.1 µg/well of the control vector (RSV-βgal) were added to each 1.5-ml microcentrifuge tube. The reaction was incubated at room temperature for approximately 45 min. Following incubation, 96 µl of the reaction (DMEM, Fugene6 and vectors) was added to each well.

Day 3. Transfected αT3-1 cells were harvested approximately 24 h later. The media was aspirated from the wells and rinsed twice with 1 ml of 1X PBS. Following the rinses, 200 µl of Lysis Buffer (Galacto-Light kit; Applied Biosystems, Bedford, MA), which contained 1 M dithiothreitol (DTT), was added to each well. The 6-well plates were incubated and shaken at 4°C for at least 10 min. The cell lysates were harvested by pipetting and washing the plates with the buffer. Next, the cell lysates and Lysis Buffer were transferred to a 0.5-ml microcentrifuge tube and centrifuged at 4°C for 2 min at 16,000 x *g* using a Spectrofuge 16M microcentrifuge (E&K Scientific, Campbell, CA). Cell lysates (20 µ1) were transferred in duplicate into a white 96-well Microflux2 plate (Thermo Labsystems, Franklin, MA) for luciferase and β-galactosidase assays. The β-galactosidase assay was incubated at room temp for 30-60 min prior to analyzing its activity using the Wallac Victor^2 instrument (Perkin Elmer). The plates were analyzed for luciferase and β-galactosidase activity utilizing the Wallac Victor^2 instrument (Perkin Elmer). Luciferase values were divided by β-galactosidase values to adjust for

transfection efficiency. Transient transfections utilized three different plasmid preparations and were completed a minimum of three times.

Protein Extraction

A Nuclear and Cytoplasmic Extraction Reagent Kit (NE-PER®; Pierce Biotechnology, Rockford, IL) was utilized to obtain nuclear protein extracts from αT3-1 cells for electrophoretic mobility shift assays (EMSAs). Four 150 mm plates of αT3-1 cells at 85% confluency were rinsed once with 10 ml of cold 1X PBS. Next, cells were removed from the plates with 10 ml of TNE buffer (10 mM Tris-Cl, 140 mM sodium chloride (NaCl), and 1 mM EDTA, pH of 8.0). Once cells were removed from the plates, they were centrifuged for 5 min at 500 x *g* and 4°C using a Beckman TJ-6 centrifuge (Beckman, Palo Alto, CA).

The supernatant was completely removed from each cell pellet. The dried cell pellet was resuspended in ice-cold CER I reagent (Pierce Biotechnology), by vortexing for 15 s, and incubated on ice for 10 min. Phosphatase Inhibitor Cocktail Set II (100X stock containing 200 mM imidazole, 100 mM sodium fluoride, 115 mM sodium molybdate, 100 mM sodium orthovanadate and 400 mM sodium tartrate dehydrate; CalBiochem, La Jolla, CA) and Protease Inhibitor Cocktail [100X stock containing 104 mM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 80 µM aprotinin, 2 mM leupeptin, 4 mM bestatin, 150 µM pepstatin A and 140 µM E-64; Sigma Chemical Co., St. Louis, MO] were added with the CER I reagent. Next, ice-cold CER II reagent (Pierce Biotechnology) was added to the cells, vortexed vigorously for 5 s and incubated

on ice for 1 min. Lysed cells were then centrifuged using a Spectrafuge 16M microcentrifuge at 16,000 x *g* (E&K Scientific, Campbell, CA) for 5 min at 4°C. The supernatant was then transferred to a clean pre-chilled 1.5-ml microcentrifuge tube and stored at -80°C. The insoluble pellet was resuspended in ice-cold NER reagent (Pierce Biotechnology) containing protease and phosphatase inhibitors. The pellet was vortexed for 15 s and then incubated on ice for a total of 40 min, vortexing for 15 s every 10 min. Lastly, the lysed nuclei were centrifuged at 16,000 x *g* for 10 min at 4°C using a Spectrafuge 16M microcentrifuge (E&K Scientific). The supernatant (nuclear extract) was then immediately transferred to a clean pre-chilled 1.5-ml microcentrifuge tube. The total protein concentration of nuclear extracts was measured using the BCA™ Assay Kit (Pierce Biotechnology). Nine standards were used for the standard curve and compared to unknown samples. A microplate (Sarstedt, Inc., Newton, NC) was used and 25 µl of each standard and unknown was loaded onto the plate in triplicate. A working reagent was prepared by mixing 50 parts of BCA™ Reagent A with 1 part of BCA™ Reagent B (50:1, Reagent A:B). The working reagent (200 µl) was added to each well and mixed thoroughly on a plate shaker for 30 s. Next, the plate was covered and incubated at 37°C for 30 min. Following incubation, the plate was cooled to room temperature and light absorbance was measured on a Spectra Max 250 plate reader (Molecular Devices, Sunnyvale, CA) at 562 nm. After the nuclear extracts were quantitated, they were stored at -80°C in 100 µl aliquots until use.

Electrophoretic Mobility Shift Assays

Oligonucleotides were annealed prior to use in EMSAs by adding 1 µl of the antisense DNA (50 µM), 1 µl of the sense DNA (50 µM), 1 µl of 10X NET buffer (1 M NaCl, 10 mM EDTA, and 100 mM Tris-Cl, pH 7.5) and Millipore water to bring to a final volume of 10 µl. The mixture was heated to 95°C for 10 min to denature the oligonucleotides, incubated at 37°C for 30 min to anneal the oligonucleotides and incubated at 25°C for an additional 30 min. Oligonucleotides had a final concentration of 5 µM and were stored at -20°C.

T4 polynucleotide kinase (PNK; Fermentas, Inc., Hanover, MD) was used to end label the oligonucleotides with [γ-^{32}P]ATP. One µl of the annealed oligonucleotide, 1 µl of 10X T4 PNK Buffer A, 4 µl of Millipore water, 1 µl of T4 PNK, and 3 µl of [γ-^{32}P]ATP were incubated together for 30 min in a 37°C water bath. After incubation, 35 µl of Millipore water was added to each reaction. MicroSpin™ G-25 columns (Amersham Biosciences Corp., Piscataway, NJ) were equilibrated by centrifuging at 3,000 x *g* for 1 min using a Mikroliter microcentrifuge (Hettich AG, Bach, Switzerland). Each reaction was added to an equilibrated MicroSpin™ G-25 column (Amersham Biosciences Corp.) and centrifuged for 2 min at 3,000 x *g* using a Mikroliter microcentrifuge (Hettich AG). Next, 5 µl of 10X NET buffer was added to each reaction. Four ml of scintillation fluid was added to 1 µl of each reaction in a scintillation vial and counted using a 1900TR liquid scintillation counter (Packard Instrument Co., Meriden, CT).

Electrophoretic mobility shift assays (EMSAs) utilized a 5% polyacrylamide gel that consisted of 6.25 ml of 38% acrylamide, 2% bisacrylamide; 5 ml of 10X TGE buffer (0.25 M tris base, 1.9 M glycine, 10 mM EDTA, pH 8.3); 150 µl of 10% ammonium persulfate, 50 µl of TEMED and Millipore water which was added to bring the final volume to 50 ml. After polymerization, the gel was pre-run at 100 V for at least 30 min in 1X TGE buffer (25 mM tris base, 190 mM glycine, and 1mM EDTA, pH 8.3). A master mix was prepared consisting of 4 µl of 2X Dignam D buffer [20 mM HEPES, pH 7.9, 20% glycerol, 0.1 M potassium chloride (KCl), 0.2 mM EDTA, 0.5 mM phenylmethanesulfonyl fluoride (PMSF; added fresh), 0.5 mM DTT (added fresh)], 1 µl of 20 mM DTT, 2 µg poly(dI·dC) (Amersham Biosciences) and 5 µg of αT3-1 nuclear extracts. The total volume was brought up to 18 µl with Millipore water and incubated on ice for 15 min. Following incubation, either 1 µl of homologous, heterologous, or consensus oligonucleotide competitors were added. Consensus oligonucleotides (Table 3.2; Integrated DNA Technologies) that were used in EMSAs included GATA, glucocorticoid receptor (GR), nuclear factor-1 (NF-1), nuclear factor-κB (NF-κB), nuclear factor-Y (NF-Y), octamer transcription factor-1 (Oct-1), mutated (µOct-1), and steroidogenic factor-1 (SF-1). Assays which utilized unlabeled competitor oligonucleotides were added with 1 µl of radiolabeled probe and the reaction was incubated at room temperature for 20 min. To determine which proteins comprised specific binding complexes, a rabbit polyclonal antibody directed against GATA-1, -2, and -4 (Santa Cruz Biotechnology, Santa Cruz, CA), a rabbit polyclonal antibody directed against the p65 (Calbiochem, La Jolla, CA) p50 (Santa Cruz Biotechnology),

TABLE 3.2. OLIGONUCLEOTIDES CONTAINING CONSENSUS TRANSCRIPTION FACTOR BINDING SITES USED AS COMPETITORS IN ELECTROPHORECTIC MOBILITY SHIFT ASSAYS[a]

Transcription Factor[b]	Binding Sequence
GATA	5'- CAC TTG ATA ACA GAA AGT GAT AAC TCT -3'
GR	5'- AGA GGA TCT GTA CAG GAT GTT CTA GAT -3'
NF-1	5'- TTT TGG ATT GAA GCC AAT ATG ATA -3'
NF-κB	5'- AGT TGA GGG GAC TTT CCC AGG C -3'
NF-Y	5'- AGA CCG TAC GTG ATT GGT TAA TCT CTT -3'
Oct-1	5'- TGT CGA ATG CAA ATC ACT AGA A -3'
μOct-1	5'- TGT CGA ATG CAA GCC ACT AGA A -3'
SF-1	5'- CAA GTT CAC CTT GAT CTT TC -3'

[a]The complement strand was annealed for each consensus oligonucleotide prior to use in gel shift assays.

[b]GR = glucocorticoid receptor; NF-1 = nuclear factor-1; NF-κB = nuclear factor-κB; NF-Y = nuclear factor-Y; Oct-1 = octamer transcription factor-1; μOct-1 = mutated octamer transcription factor-1; SF-1 = steroidogenic factor-1.

and p52 (Upstate, Charlottesville, VA) subunits of NF-κB, polyclonal SF-1 antibodies (gifted by Dr. Ken-ichirou Morohashi, National Institute for Basic Biology, Okazaki, Japan), a rabbit polyclonal antibody directed against Oct-1 (Santa Cruz Biotechnology), or an equal mass of rabbit IgG (Santa Cruz Biotechnology) was used. Prior to addition of the probe labeled with [γ-^{32}P]ATP, the master mix was incubated with GATA antibodies for 2 h at 4°C, NF-κB antibodies for 30 min at room temperature, SF-1 antibodies for 2 h at room temperature, or Oct-1 antibodies for 1 h at room temperature. After addition of the radiolabeled probe, the reactions were incubated at room temperature for 20 min. Reaction samples were then loaded to the polyacrylamide gel subjected to electrophoresis at 40 mA for approximately 1.5 hours. Next, the polyacrylamide gel was transferred to blotting paper (3 mm; Whatman, Maidstone, England), dried on a Fisherbiotech Gel Dryer Model FB-GD-45 (Fisher Scientific, Pittsburgh, PA) and exposed to Blue Sensitive Radiographic Film (Marsh Bio Products, Inc., Rochester, NY) for 20-24 h at -80°C. The film was later developed using an SRX-101A medical film developer (Konica Corp., Wayne, NJ).

Statistical Analysis

Data were analyzed using analysis of variance (ANOVA) within the general linear models (GLM) procedure of the Statistical Analysis System (SAS, 2001). Least significant differences was used to compare means for luciferase activity among test vectors. Means for luciferase activity of test vectors were compared with control vectors using Dunnett's *t*-test.

CHAPTER IV

Three Steroidogenic Factor-1 Binding Sites Confer Gonadotrope-Specific Activity Whereas GATA-4 and Nuclear Factor-κB Elements Contribute to Divergent Line-Specific Activity of the Porcine GnRH Receptor Gene Promoter

Abstract

The interaction between GnRH and its receptor is critical for regulation of reproductive function. Additionally, the porcine GnRH receptor (GnRHR) gene is located near a quantitative trait locus (QTL) for ovulation rate. Thus, the GnRHR gene represents both a physiological and positional candidate for genes influencing ovulation rate. Transient transfection of gonadotrope-derived αT3-1 cells with luciferase reporter constructs containing the GnRHR promoter from 3 swine lines with divergent ovulation rates (Control, Index or Meishan) revealed gonadotrope-specific activity that was significantly elevated for the Meishan promoter. Our first objective was to identify elements within an important distal fragment, the swine upstream promoter enhancing region (SUPER; -1779/-1667), conferring cell-specific activity of the porcine GnRHR promoter. Electrophoretic mobility shift assays (EMSA) using αT3-1 nuclear extracts, radiolabeled oligonucleotides spanning SUPER and an antibody directed against SF-1, revealed a binding site for SF-1 at -1760/-1753 bp of proximal promoter. Transient transfection of αT3-1 cells with reporter vectors containing a block replacement mutation of the SF-1 binding site within the context of the full-length Control promoter reduced luciferase activity by approximately 30% compared to the full-length Control promoter

($P < 0.05$). Previously, transient transfection assays indicated that additional line-specific elements may lie between -1000 and -500 bp of 5' flanking region for the porcine GnRHR gene. Therefore, our second objective was to identify line-specific elements within both the -1000/-500 bp promoter region and SUPER. Sequence analysis of the -1000/-500 fragment identified a bp substitution (-845 bp) unique to the Meishan GnRHR gene promoter. Inclusion of a GATA-4 antibody resulted in a supershift of the DNA:protein complex confirming that GATA-4 binds to the Meishan GnRHR promoter. Transient transfection of αT3-1 cells with a vector containing a block replacement mutation of the GATA-4 element reduced luciferase activity by approximately 20% ($P < 0.05$) compared to the full-length Meishan promoter. Another single bp substitution within SUPER (-1690 bp) allowed GATA-4 to bind to the Meishan promoter, whereas the p65/p52 subunits of NF-κB bound to the homologous Control/Index promoters. Transient transfection of αT3-1 cells with vectors containing the block replacement mutations of either the GATA-4 or NF-κB binding sites within the context of their native promoters resulted in a 50 and 60% reduction of luciferase activity, respectively. Thus, a bp alteration (-845) in the Meishan GnRHR promoter recruits unique binding factors whereas SUPER contains both cell- and line-specific elements, contributing to the enhanced activity of the Meishan promoter.

Introduction

Gonadotropin-releasing hormone (GnRH) is a decapeptide hormone that is released in a pulsatile manner from the hypothalamus and travels through the

hypothalamo-hypophoseal portal system to the anterior pituitary gland (Carmel et al., 1976; Levine et al., 1982). Gonadotropin releasing hormone binds to its cognate receptor on the plasma membrane of gonadotrope cells within the anterior pituitary gland, resulting in the synthesis and secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH; Clayton and Catt, 1981; Clarke et al., 1983). Luteinizing hormone is responsible for ovulation in the female and testosterone production in the male, whereas FSH is important for follicular recruitment and development in the female and spermatogenesis in the male. Furthermore, GnRH binding to its G-coupled protein receptor (GPCR) stimulates the up-regulation of at least 4 gonadotropic genes: the common glycoprotein α-subunit, the LH-β and FSH-β subunits (Hamernik and Nett, 1988; Gharib et al., 1990), and the GnRH receptor (GnRHR) itself (Sealfon and Millar, 1995). The gonadotropins, in turn, act on the gonads to cause the production of steroid hormones. The steroid hormones can act in a negative feedback loop at the level of the anterior pituitary and hypothalamus to control the production of the gonadotropins and GnRH, respectively (Nakai et al., 1978; Conn and Crowley, 1994; McNeilly et al., 2003). Thus, the interaction between GnRH and its receptor represents a critical point for regulation of reproduction function in mammals.

The GnRHR gene promoter has been previously isolated and studied in various species including the mouse, rat and human. The mouse GnRHR gene promoter was the first to be isolated and analyzed (Tsutsumi et al., 1992; Clay et al., 1995). Cell-specific activity of the mouse GnRHR gene promoter is regulated by a tripartite basal enhancer that consists of 3 elements; steroidogenic factor-1 (SF-1; -244/-236 bp), activating

protein-1 (AP-1; -336/-330 bp) and a GnRHR activating sequence (GRAS; -391/-380 bp; Duval et al., 1997a, 1997b). Each of these elements contribute equally to activity of the mouse GnRHR promoter. Steroidogenic factor-1 is an orphan member of the nuclear receptor superfamily that is common to promoters of all gonadotropic genes and the AP-1 element confers GnRH responsiveness of the mouse GnRHR gene (Norwitz et al., 1999; White et al., 1999). In addition, GRAS regulates activin responsiveness of the mouse GnRHR gene promoter. The functional activity of GRAS is dependent on the organization of a multi-protein complex which is made up of Smad3/4, AP-1, and a member of the forkhead family of DNA binding proteins, FoxL2 (Ellsworth et al., 2003a). In addition to the tripartite enhancer, another enhancer element critical to maximal GnRH-stimulated activity of the mouse GnRHR gene, termed the sequence underlying responsiveness to GnRH-1 (SURG-1; Norwitz et al., 1999), was identified.

The rat GnRHR gene promoter is regulated by the same tripartite enhancer as the mouse. However, the rat promoter possesses a distal enhancer, GnRHR specific enhancer (GnSE), which also contributes to cell-specific expression of the rat GnRHR gene (Pincas et al., 2001). This distal GnSE contains binding sites for GATA and LIM homeodomain-related factors and also interacts with an SF-1 recognition site (Pincas et al., 1998; Pincas et al., 2001). Despite characterization of placental-, granulosa/luteal cell- and neuronal-specific promoters as well as identification of elements responsive to GnRH, cAMP, P_4 and E_2 (Cheng and Leung, 2005), functional analysis of the 5' flanking region for the human GnRHR gene has only revealed an SF-1 binding site required for gonadotrope-specific expression (Ngan et al., 1999). Interestingly, prolonged treatments

with GnRH indicated that an upstream AP-1 element is involved in transcriptional down-regulation of the GnRHR gene (Cheng et al., 2000b) and an Oct-1 binding site has been shown to act as a constitutive repressor of GnRHR gene transcription (Cheng et al., 2002b).

In addition to its established physiological importance, the porcine GnRHR gene is located in close proximity to a quantitative trait locus (QTL) associated with ovulation rate on chromosome 8 (Rohrer et al., 1999). Therefore, the porcine GnRHR gene represents both a physiological and positional candidate for genes influencing ovulation rate. To understand the relationship between the GnRHR gene and ovulation rate, our laboratory has utilized 3 swine lines with divergent ovulations rates; a Control white-crossbred line, an Index line and the Chinese Meishan. The Index line was selected for over 14 generations based on an index of embryonic survival and ovulation rate (Johnson et al., 1984). These females ovulate 7 more oocytes per estrous cycle compared to the Control line, resulting in 1.4 more piglets per litter (Johnson et al., 1999). Chinese Meishan females produce approximately 4 to 5 more pigs per litter than females from occidental breeds, largely due to an increased ovulation rate (Christenson et al., 1993; White et al., 1993).

Identification of mutations within gene promoters that are correlated with enhanced physiological or quantitative traits is not unprecedented. The porcine corticotropin-releasing hormone (CRH) gene is located in proximity to QTLs for growth and carcass composition on chromosome 4 and therefore, is a positional candidate for genes associated with these traits (Murani et al., 2006). A single bp alteration within the

promoter of the CRH gene had no significant effect on growth and carcass composition in swine lines, but it may contribute to differences in meat quality among lines (Murani et al., 2006). Several polymorphisms within the promoters of genes specific to the mammary gland in dairy cattle have positive effects on milk production. Kuss et al. (2005) identified a single bp substitution in the bovine α_{s1}-casein-encoding gene promoter. Animals harboring this polymorphism displayed higher milk content traits and increased amounts of α_{s1}-casein in their milk. Furthermore, the bovine acyl-CoA:diacylglycerol acyltransferase1 (DGAT1) gene contains several polymorphisms in German Angeln dairy cattle resulting in higher milk yield traits compared to other breeds (Sanders et al., 2006).

In 1994, Weesner and Matteri sequenced the cDNA for the porcine GnRHR gene. Jiang and associates (2001) identified 1154 bp of proximal promoter for the porcine GnRHR gene. Based on this sequence, our laboratory has isolated 5118 bp of 5' flanking region for the porcine GnRHR gene promoter from the Control, Index and Meishan lines of swine and constructed luciferase reporter vectors containing each of these promoters. Transient transfection of reporter vectors into the gonadotrope-derived αT3-1 cell line revealed divergent luciferase activity among the 3 swine lines (McDonald, 2005). We have also established polymorphisms within the 5' flanking sequence of the GnRHR genes among swine lines. Previously, we identified a single bp alteration unique to the Meishan located at -1235 bp of 5' flanking region that allows the p65 and p52 subunits of NF-κB and an Sp-1 like protein to bind to the Meishan GnRHR gene promoter but not the Control promoter (McDonald, 2005). In addition, transient transfection assays indicated

that additional line-specific elements may lie between -1000 and -500 bp of 5' flanking region for the porcine GnRHR gene. Recently, we identified a critical upstream enhancer region, termed the swine upstream promoter enhancing region (SUPER), located at -1779/-1667 bp that contributed to cell-specific activity of the porcine GnRHR gene promoter (Cederberg et al., unpublished data). Therefore, the objectives of this study were to identify line-specific elements within the -1000/-500 bp promoter region as well as cell- and line-specific elements within the recently identified SUPER that contribute to activity of the porcine GnRHR gene promoter.

Materials and Methods

Materials. The antibody directed against the p65 subunit of NF-κB (catalog no. PC137) was purchased from Calbiochem (La Jolla, CA) and the antibody specific for the p52 subunit of NF-κB (catalog no. 06-413) was from Upstate (Charlottesville, VA). The specific antibodies for the p50 subunit of NF-κB (catalog no. sc-114X), GATA-1 (catalog no. sc-1234X), GATA-2 (catalog no. sc-9008X), GATA-4 (catalog no. sc-1237), octamer transcription factor-1 (Oct-1; catalog no. sc-232X) and normal rabbit IgG (catalog no. sc-2027) were all obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The SF-1 antibody was gifted by Dr. Ken-ichirou Morohashi (National Institute for Basic Biology, Okazaki, Japan). For experiments using electrophoretic mobility shift assays (EMSAs), competitive oligonucleotides containing consensus binding sites for GATA, glucocorticoid receptor (GR), nuclear factor-1 (NF-1), NF-κB, nuclear factor-Y (NF-Y),

Oct-1, mutated (μOct-1) or SF-1 (See Table 3.2) were synthesized by Integrated DNA Technologies (Coralville, IA).

Plasmids. Using primers specific for the porcine GnRHR gene promoter originally isolated from the Control line (Cederberg et al., unpublished data), we identified promoters from genomic DNA of the Meishan and Index lines. Full-length GnRHR gene promoters (-5118) from the three genetic pig lines were sub-cloned into the pGL3 basic reporter vector (Promega Corp., Madison, WI). Plasmids harboring block replacement mutations were comprised of 5118 bp of 5' flanking sequence for the GnRHR gene with individual elements mutated to contain: a *PstI* site replacing the GATA binding site at -845/-840 bp (Mμ845GATA4pGL3) or a *Bcu/SpeI* site substituted for the GATA element at -1694/-1689 bp (Mμ1690GATA4pGL3) within the Meishan promoter; an *EcoRI* site in place of the NF-κB binding site at -1699/-1694 bp (C/Iμ1699NFκBpGL3) or an *EcoRI* site replacing the NF-κB element at -1689/-1684 bp (C/Iμ1689NF-κBpGL3) within the Control and Index promoters; and a *NotI* (μSF-1pGL3) or *SacI* (μOct-1pGL3) site in exchange for the SF-1 (-1760/-1753) or Oct-1 (-1731/-1724) binding sites, respectively, within the promoters for all 3 swine lines. Overlap extension PCR mutagenesis was performed through two rounds of PCR in order to specifically mutate the binding element of interest. The first round of PCR utilized primers replacing the binding site of interest with a restriction site, and the second round used product from the first round as template to anneal and replicate the mutated element and flanking sequence. To verify that the correct mutations had been introduced, vectors were sequenced at the University of Nebraska-Lincoln Genomics Core Research Facility

before use in transient transfection experiments. The vector used as a control for transfection efficiency in all experiments contained the Rous sarcoma virus promoter fused to the cDNA encoding β-galactosidase (RSV-β-gal, Stratagene, La Jolla, CA). A Plasmid Midi Kit (Qiagen, Valencia, CA) was used to isolate transfection quality DNA.

Cell Culture and Transient Transfections. Cultures of αT3-1 cells (Dr. Pam Mellon, Salk Institute, La Jolla, CA) were maintained at 37° C in a humidified 5% CO2 in air atmosphere. The αT3-1 cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM; Mediatech, Herndon, VA) supplemented with 5% fetal bovine serum, 5% horse serum, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin sulfate (Gibco, Grand Island, NY). Transient transfections were carried out using a liposome-mediated protocol (Fugene6, Roche Diagnostics Corp., Indianapolis, IN) according to manufacturer's instructions. Briefly, 2 x 10^6 cells were plated in 6-well culture dishes the day prior to transfection. Cells were transfected with a 3:1 Fugene6 to DNA ratio. A total of 1 μg of DNA, 0.9 μg of luciferase test vector and 0.1 μg of RSV-β-gal control vector, were used per well. Approximately 20-24 h post-transfection, cells were washed twice with ice-cold 1X PBS and harvested with 200 μl of lysis buffer [Tropix Lysis Buffer, Applied Biosciences, Bedford, MA and 1 mM dithiothreitol (DTT)]. Lysates were cleared by centrifugation at 16,000 x *g* for 2 min at 4° C. Lysates (20 μl) were immediately analyzed according to manufacturer's instructions for both luciferase (Promega Corp.) and β-gal (Tropix, Applied Biosystems, Bedford, MA) activity using a Wallac Victor^2 microplate reader (PerkinElmer Life Sciences, Boston,

MA). Luciferase values were divided by β-gal values to adjust for transfection efficiency.

Electrophoretic Mobility Shift Assays. Nuclear protein extracts were obtained from approximately 2.8 x 10^8 αT3-1 cells using the NE-PER® Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce Biotechnology, Rockford, IL). The nuclear extracts were treated with protease (catalog no. P8340, Sigma Chemical Co., St. Louis, MO) and phosphatase (catalog no. 524625, Calbiochem, La Jolla, CA) inhibitor cocktail solutions to prevent enzymatic degradation of proteins. The amount of protein present in the extracts was determined using a bicinchoninic acid (BCA) Protein Assay (Pierce). Oligonucleotides were end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (Fermentas Inc., Hanover, MD) and purified using sephadex G-25 spin columns (Amersham Biosciences Corp., Piscataway, NJ). Nuclear extracts (5 μg) were incubated in 20 μl reactions containing 2X Dignam D buffer [20 mM HEPES, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF (added fresh), 0.5 mM DTT (added fresh)], 20 mM DTT, 2 μg of poly(dI•dC) (Amersham Biosciences) and, where indicated, a rabbit polyclonal antibody directed against the p65, p52 and p50 subunits of NF-κB, GATA-1, -2 and -4, Oct-1, SF-1, or an equal amount of rabbit IgG. Prior to addition of the radiolabeled probe, the master mix was incubated with GATA antibodies for 2 h at 4°C, NF-κB antibodies for 30 min at room temperature, SF-1 antibodies for 2 h at room temperature, or Oct-1 antibodies for 1 h at room temperature. Following incubation, radiolabeled probe (100,000 cpm) and 50-fold molar excess of either homologous or

heterologous unlabeled competitor was added. Where indicated, 50-fold molar excess of unlabeled oligonucleotides containing consensus binding sequences for GATA, GR, NF-1, NF-κB, NF-Y, Oct-1, μOct-1, or SF-1 were also added. The final reactions were incubated at 25° C for 20 min before bound probe was separated from free probe at 40 mA for 1.5 h on a 5% polyacrylamide gel that had been pre-run at 100 V for 1 h in 1X TGE [25 mM tris base, 190 mM glycine and 1 mM EDTA, pH 8.3]. Gels were transferred to blotting paper, dried, and exposed to Blue Sensitive Radiograph film (Marsh Bio Products Inc., Rochester, NY) for 20-24 h at -80° C before being developed in a SRX-101A medical film developer (Konica Corp., Wayne, NJ).

Statistical Analysis. Data were analyzed using analysis of variance (ANOVA) within the general linear models (GLM) procedure of the Statistical Analysis System (SAS, 2001). Least squares means for luciferase activity were compared among test vectors using least significant differences. Means for luciferase activity of test vectors were compared with control vectors using Dunnett's *t*-test. All transfections were performed a minimum of three times using different plasmid preparations. Additionally, each plasmid was transfected in triplicate wells within each transfection.

Results

A single bp alteration unique to the Meishan GnRHR gene promoter allows for divergent binding of GATA-4. Sequence analysis of the -1000 to -500 5' flanking region revealed 4 single bp alterations among the 3 pig lines. Electrophoretic mobility shift assays were performed using αT3-1 nuclear extracts and radiolabeled

oligonucleotides spanning the 4 single bp substitutions identified between -1000 and -500 bp of proximal promoter to determine if differential binding existed among the swine lines. Only the oligonucleotide representing the bp substitution at -845 bp formed a specific binding complex in the Meishan oligonucleotide, whereas binding was absent in the Control/Index oligonucleotides (Figure 4.1A). Addition of unlabeled oligonucleotides containing consensus binding sites for GR, NF-1 or GATA resulted in ablated binding of the specific complex by the consensus GATA oligonucleotide, indicating that a member of the GATA family of transcription factors comprised the binding complex (Figure 4.1A). Addition of an antibody directed against GATA-4, but not GATA-1 or -2, induced a supershift of the DNA:protein complex (Figure 4.1B). Therefore, GATA-4 binds to the sequence spanning the bp substitution at -845 bp of the Meishan GnRHR gene promoter and represents an important factor for line-specific activity of the Meishan promoter.

Block replacement mutation of the GATA-4 binding site located at -845 bp of proximal promoter reduced luciferase activity of the Meishan GnRHR gene promoter. To determine the functional significance of the GATA-4 binding site unique to the Meishan promoter, αT3-1 cells were transiently transfected with luciferase reporter vectors containing either the full length Meishan promoter (M5000pGL3), a block replacement mutation of the GATA-4 binding site within the context of the full-length Meishan promoter (Mμ845GATA-4pGL3) or promoterless control (pGL3). Reporter vectors containing the GATA-4 block replacement mutation (Mμ845GATA-4pGL3)

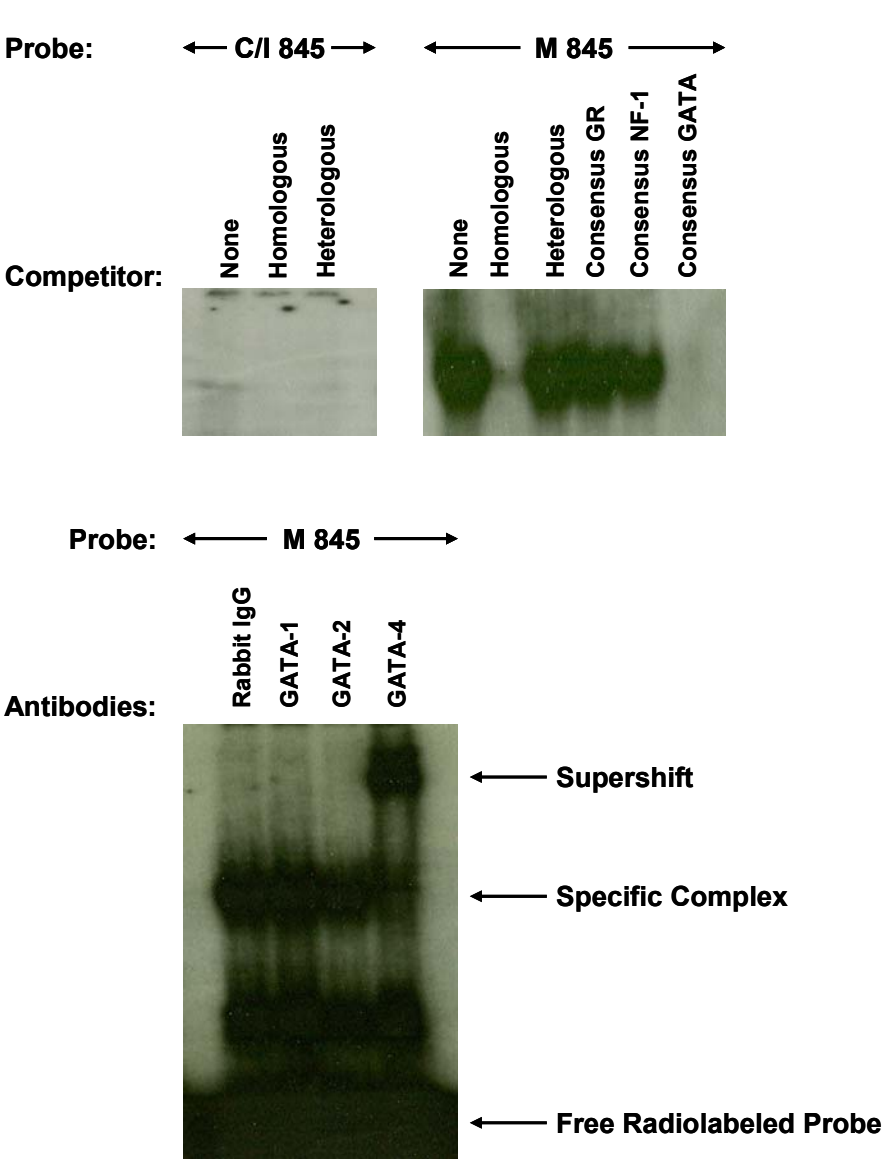


Figure 4.1. The transcription factor, GATA-4, comprises the specific complex binding to the sequence spanning a bp substitution (-845) unique to the Meishan GnRHR gene promoter. (A) Electrophoretic mobility shift assays were performed by incubating αT3-1 nuclear extracts with radiolabeled oligonucleotides spanning the bp alteration at -845. Specificity of the DNA:protein complex was tested by the addition of 50-fold molar excess of either unlabeled homologous or heterologous DNA. In addition, 50-fold molar excess of unlabeled oligonucleotides containing consensus binding sequences for GR, NF-1 or GATA were added to the reaction. (B) To determine the specific proteins which comprised the DNA:protein complex binding to the oligonucleotide spanning the substitution at -845 bp, antibodies directed against GATA-1, -2 and -4 or an equal mass of rabbit IgG were added.

reduced luciferase activity ($P < 0.05$) by approximately 20% compared to the native Meishan promoter (Figure 4.2). Despite the reduced luciferase activity in cells transfected with Mμ845GATA-4pGL3, these values still remained numerically higher than those obtained for the full-length Control promoter (15- to 25-fold over promoterless control) in previous transfections. This suggests that additional unidentified element(s) within the Meishan proximal promoter contribute to its enhanced activity.

Binding of SF-1 to a recognition site located at -1760/-1753 bp of proximal promoter represents the first factor comprising the swine upstream promoter enhancing region (SUPER). A transcription factor binding site search identified a putative SF-1 binding site located within SUPER. An oligonucleotide spanning the SF-1 binding site located at -1760/-1753 bp of 5' flanking region of the porcine GnRHR gene promoter was used in EMSAs. Incubation of radiolabeled oligonucleotide with αT3-1 nuclear extracts and the addition of either homologous or heterologous DNA identified a specific binding complex (Figure 4.3). Inclusion of unlabeled oligonucleotides containing consensus binding sites for NF-Y or SF-1 resulted in competition by the consensus SF-1 oligonucleotide (Figure 4.3). Addition of an antibody directed against the DNA binding domain of SF-1 ablated the DNA:protein complex (Figure 4.3). Thus, SF-1 interacts with the binding site (-1760/-1753) located within SUPER, an important distal enhancer of the GnRHR gene promoter.

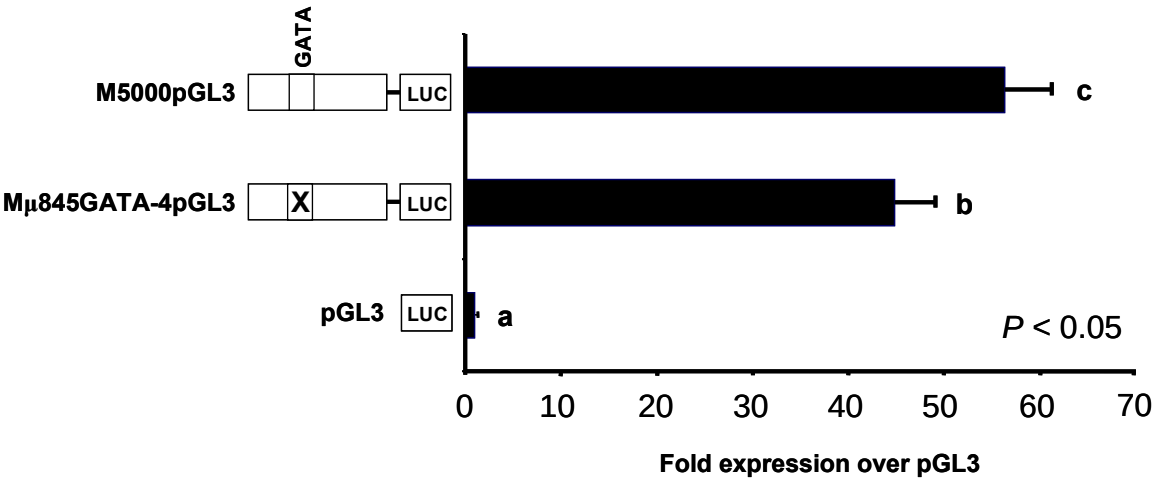


Figure 4.2. Functional significance of the GATA-4 binding site specific to the Meishan GnRHR gene promoter. Transient transfections of αT3-1 cells with luciferase (LUC) reporter vectors containing the native, full-length Meishan promoter (M5000pGL3), a block replacement mutation of the GATA-4 binding site within the context of the full-length Meishan promoter (Mμ845GATA-4pGL3) or promoterless control (pGL3) were performed. Cells were harvested 24 h following transfection and assayed for LUC and β-galactosidase (β-gal) activity. Luciferase values were divided by β-gal values to adjust for transfection efficiency. Differences between vectors are indicated by bars with unique letters ($P < 0.05$).

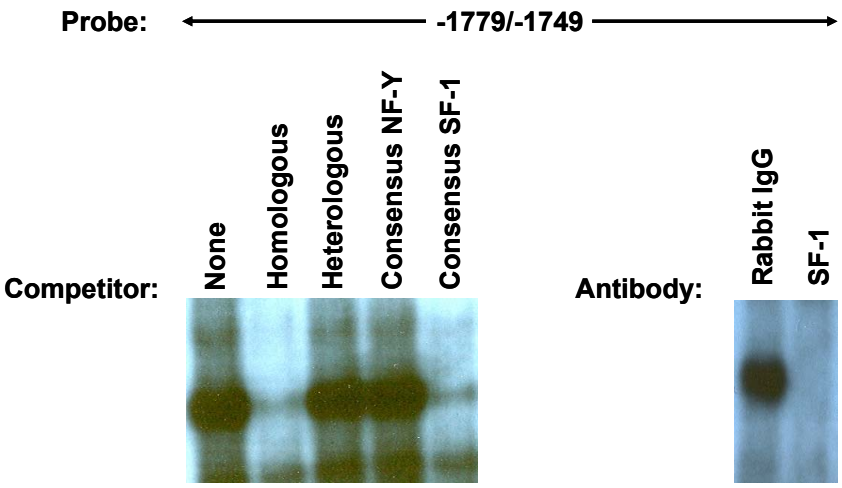


Figure 4.3. The oligonucleotide spanning the region located at -1779/-1749 bp of the porcine GnRHR gene promoter binds SF-1 in αT3-1 cells. Electrophoretic mobility shift assays were performed by incubating αT3-1 nuclear extracts with radiolabeled oligonucleotides spanning -1779/-1749 bp of proximal promoter. Specificity of the DNA:protein complex was tested by the addition of 50-fold molar excess of either unlabeled homologous or heterologous DNA. Next, 50-fold molar excess of unlabeled oligonucleotides containing consensus binding sequences for NF-Y or SF-1 were added. To determine the specific protein which comprised the binding complex, an antibody directed against the DNA binding domain of SF-1 or an equal mass of rabbit IgG were included.

The SF-1 binding site within SUPER is functionally significant to the porcine GnRHR gene promoter. Luciferase reporter vectors containing the native full-length Control promoter (C5000pGL3), a block replacement mutation of the SF-1 binding site (μSF-1pGL3) or promoterless control (pGL3) were transiently transfected into αT3-1 cells. Cells containing the block replacement mutation vector of the SF-1 binding site (μSF-1pGL3) resulted in approximately a 30% reduction in luciferase activity ($P < 0.05$) compared to cells transfected with the full-length Control promoter (Figure 4.4). Therefore, this SF-1 binding site represents an additional distal member of the cell-specific promoter for the porcine GnRHR gene. Two functional SF-1 binding sites located at -315/-307 and -179/-171 bp of proximal promoter have previously been identified (Cederberg et al., unpublished data). In addition, identification of this element allowed us to further refine the boundaries of SUPER from -1779/-1667 to -1760/-1667 bp upstream of the translational start site for the porcine GnRHR gene.

Putative Oct-1 and NF-κB elements identified within SUPER do not contribute to functional activity of the porcine GnRHR gene promoter. Putative Oct-1 and NF-κB binding sites were identified within SUPER. The NF-κB element was specific to the homologous Control/Index promoters, whereas the Oct-1 binding site was present in all 3 swine lines. Electrophoretic mobility shift assays utilizing αT3-1 nuclear extracts and radiolabeled oligonucleotides spanning the NF-κB binding site located at -1689/-1684 bp of proximal promoter formed a specific binding complex (Data not shown). Inclusion of antibodies specific for the p65, p50 and p52 subunits of NF-κB

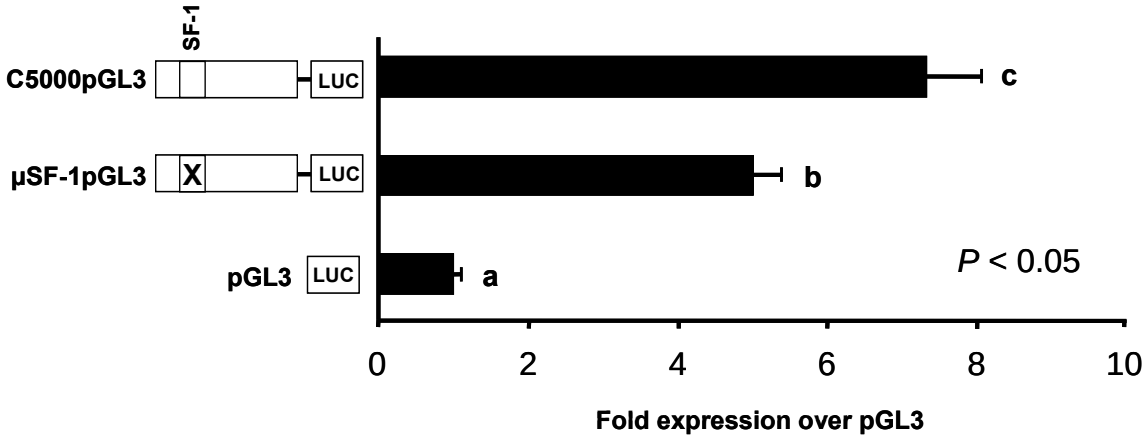


Figure 4.4. Functional significance of the SF-1 binding site within the porcine GnRHR gene promoter. Transient transfections of αT3-1 cells with luciferase (LUC) reporter vectors containing the native, full-length Control promoter (C5000pGL3), a block replacement mutation of the SF-1 binding site within the context of the full-length Control promoter (μSF-1pGL3) or promoterless control (pGL3) were performed. Cells were harvested 24 h following transfection and assayed for LUC and β-galactosidase (β-gal) activity. Luciferase values were divided by β-gal values to adjust for transfection efficiency. Differences between vectors are indicated by bars with unique letters ($P < 0.05$).

resulted in a supershift of the p65 and p52 subunits of NF-κB (See Appendix I, Panel A). In addition, EMSAs using αT3-1 nuclear extracts and a radiolabeled oligonucleotide spanning the Oct-1 element located at -1731/-1724 bp of 5' flanking region revealed a specific binding complex (See Appendix II, Panel A). However, αT3-1 cells transiently transfected with luciferase reporter vectors containing block replacement mutation vectors for either the NF-κB (C/Iμ1689NF-κBpGL3) or Oct-1 (μOct-1pGL3) elements did not differ in luciferase activity compared to the full-length porcine GnRHR gene promoter (See Appendices I and II, Panel B). Thus, despite recruitment of binding factors in αT3-1 cells, the Oct-1 and NF-κB elements were not functionally relevant to cell- or line-specific activity of the porcine GnRHR gene promoter.

A single bp substitution within SUPER confers divergent binding between the Meishan and Control/Index GnRHR gene promoters. Sequence analysis identified a single bp alteration located within SUPER at -1690 bp of proximal promoter between the Meishan and homologous Control/Index promoters. Electrophoretic mobility shift assays utilizing αT3-1 nuclear extracts and radiolabeled oligonucleotides spanning the bp substitution (-1690) between the Meishan and Control/Index promoters revealed a specific binding complex for both swine lines (Figure 4.5A). Sequence analysis of this region identified potential NF-κB and GATA elements in the Meishan and Control/Index promoters. Addition of antibodies directed against the p65 and p52 subunits of NF-κB resulted in a supershift of the DNA:protein complex for the Control/Index oligonucleotide. However, the specific binding complex associated with

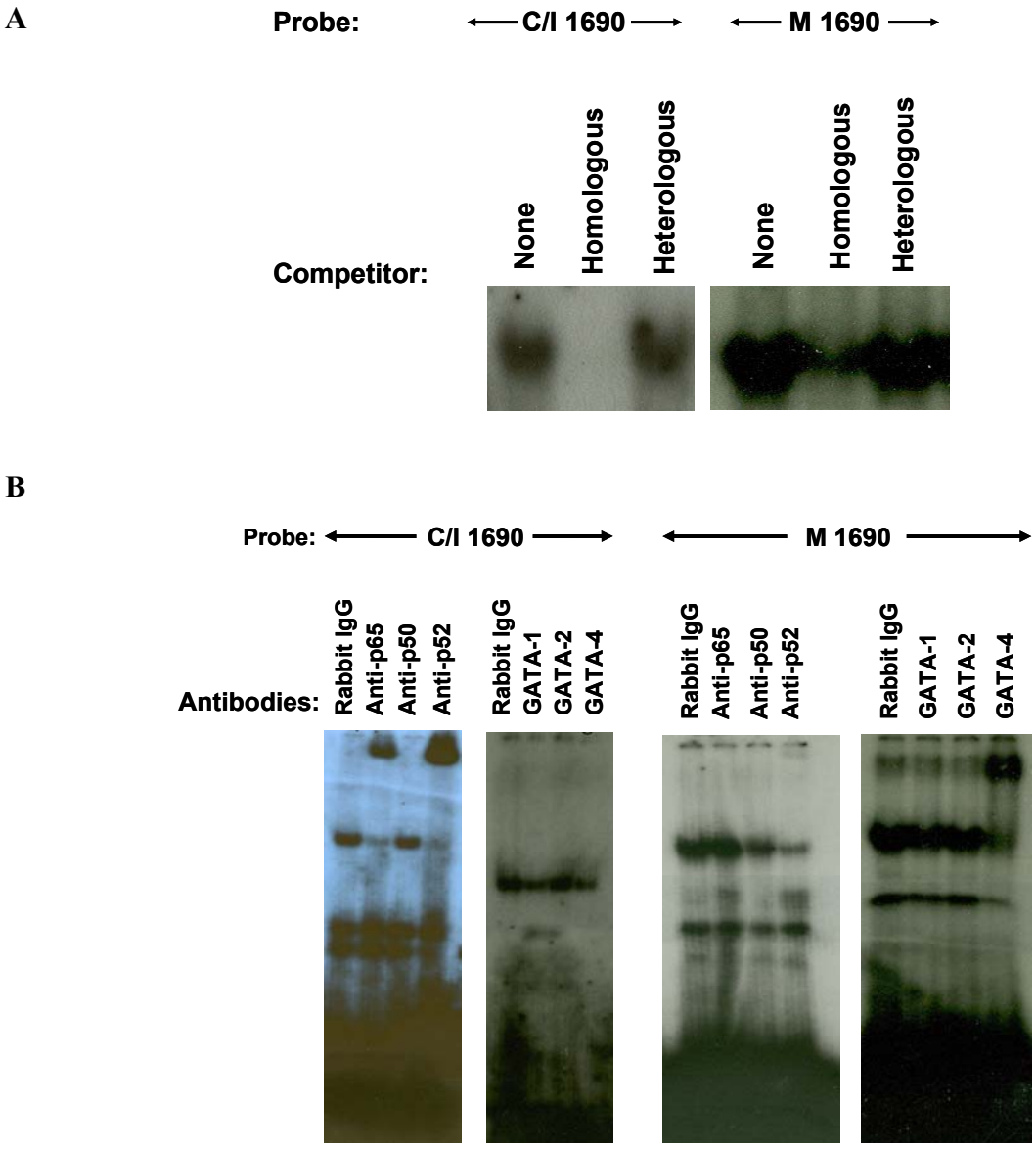


Figure 4.5. The p65 and p52 subunits of NF-κB bind to the homologous Control/Index promoters, whereas GATA-4 binds to the Meishan promoter within the 5' flanking region spanning the -1690 bp substitution. Electrophoretic mobility shift assays were performed with αT3-1 nuclear extracts and radiolabeled oligonucleotides spanning the -1690 bp substitution between the Control/Index and Meishan swine lines. A) To determine the specificity of the DNA:protein complex, 50-fold molar excess of either unlabeled homologous or heterologous DNA were added. B) To determine specific proteins which comprised the DNA:protein complex binding to the oligonucleotide spanning the substitution at -1690 bp, antibodies directed against the p65, p50, and p52 subunits of NF-κB, GATA-1, -2 and -4, or an equal mass of rabbit IgG were added.

the Meishan oligonucleotide was supershifted by an antibody specific for GATA-4 (Figure 4.5B). Therefore, the single bp alteration in the Meishan promoter forms a GATA element that allows GATA-4 binding instead of the p65 and p52 subunits of NF-κB which bind to the Control/Index promoters.

Block replacement mutations of the GATA-4 and NF-κB binding sites in the Meishan and Control/Index promoters, respectively, reduced luciferase activities of both the Meishan and Control/Index GnRHR gene promoters. Transient transfections of αT3-1 cells with luciferase reporter vectors containing either the native, full-length Meishan (M5000pGL3) and Control (C5000pGL3) promoters, block replacement mutations of the GATA-4 (Mμ1690GATA-4pGL3) and NF-κB (C/Iμ1699NF-κBpGL3) binding sites within the context of their respective full-length promoter or promoterless control (pGL3) were performed. Reporter vectors containing the block replacement mutation of the GATA-4 binding site (Mμ1690GATA-4pGL3) resulted in approximately a 50% reduction in luciferase activity ($P < 0.05$) compared to the native Meishan promoter. Further, αT3-1 cells transfected with the reporter vectors containing the block replacement mutation of the NF-κB binding site (C/Iμ1699NF-κBpGL3) reduced luciferase activity ($P < 0.05$) by approximately 60% compared to cells containing the native Control promoter (Figure 4.6). Thus, this single bp substitution contributes to the line-specific activity of the porcine GnRHR gene promoter.

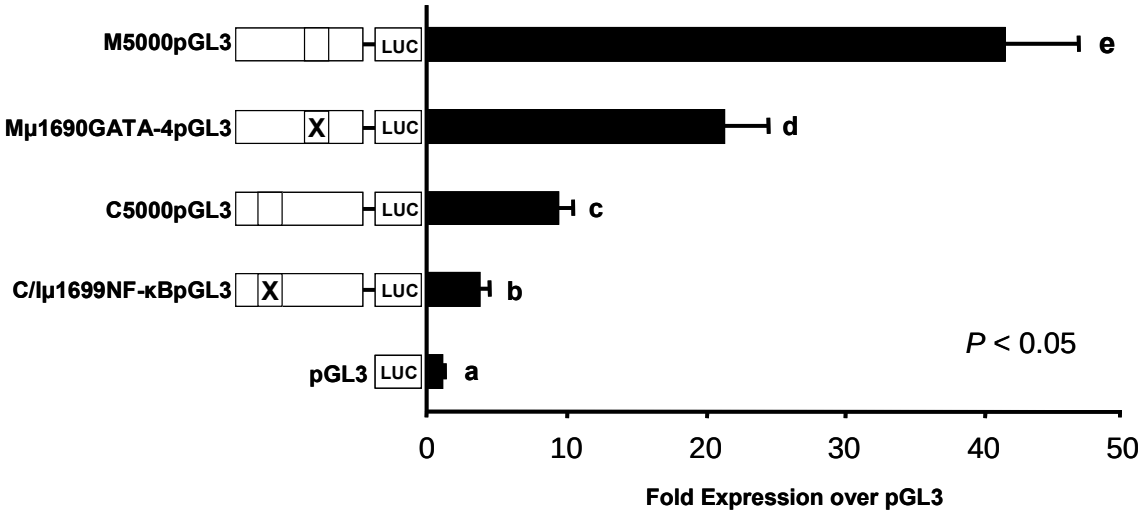


Figure 4.6. Functional significance of the GATA-4 and NF-κB elements specific to the Meishan and Control/Index GnRHR gene promoters, respectively. To confirm the importance of the GATA-4 binding site within the Meishan promoter and the NF-κB element within the Control/Index promoters, block replacement mutations of each binding site were constructed within the context of their respective full length promoter. Transient transfections of αT3-1 cells with luciferase (LUC) reporter vectors containing both full-length promoters (M5000pGL3 and C5000pGL3), block replacement mutations of the GATA-4 (Mμ1690GATA-4pGL3) and NF-κB (C/Iμ1699NF-κBpGL3) elements or promoterless control (pGL3) were performed. Cells were harvested 24 h following transfection and assayed for LUC and β-galactosidase (β-gal) activity. Luciferase values were divided by β-gal values to adjust for transfection efficiency. Differences between vectors are indicated by bars with unique letters ($P < 0.05$).

The working models for cell- and line-specific activity of the porcine GnRHR gene promoter have been updated as a result of these studies. In addition to the proximal SF-1 binding sites located at -315/-307 and -179/-171 bp of proximal promoter, an additional distal SF-1 element located at -1760/-1753 bp of 5' flanking region can be added to the cell-specific promoter of the porcine GnRHR gene (Figure 4.7A). This is a unique result because no previously identified gonadotropic gene promoters are regulated by 3 SF-1 binding sites. In addition to the cell-specific activity of the porcine GnRHR gene promoter, we can also add to our working model for line-specific activity. Our laboratory previously identified a single bp substitution unique to the Meishan promoter located at -1235 bp relative to the translational start site of the porcine GnRHR gene. This bp alteration allows the p65 and p52 subunits of NF-κB and an Sp1-like protein to bind to the Meishan promoter, whereas they do not bind to the homologous Control/Index promoters (Figure 4.7B). Another bp substitution located at -845 was identified in the Meishan promoter. This single bp alteration allows GATA-4 to bind to the Meishan promoter, whereas it is absent in the Control/Index promoters (Figure 4.7B). Furthermore, an additional distal polymorphism (-1690) within SUPER allows GATA-4 to bind to the Meishan promoter, whereas the p65 and p52 subunits of NF-κB bind to the homologous Control/Index promoters (Figure 4.7B). Therefore, the 3 identified elements unique to the Meishan promoter contribute to its enhanced activity over the Control/Index promoters.

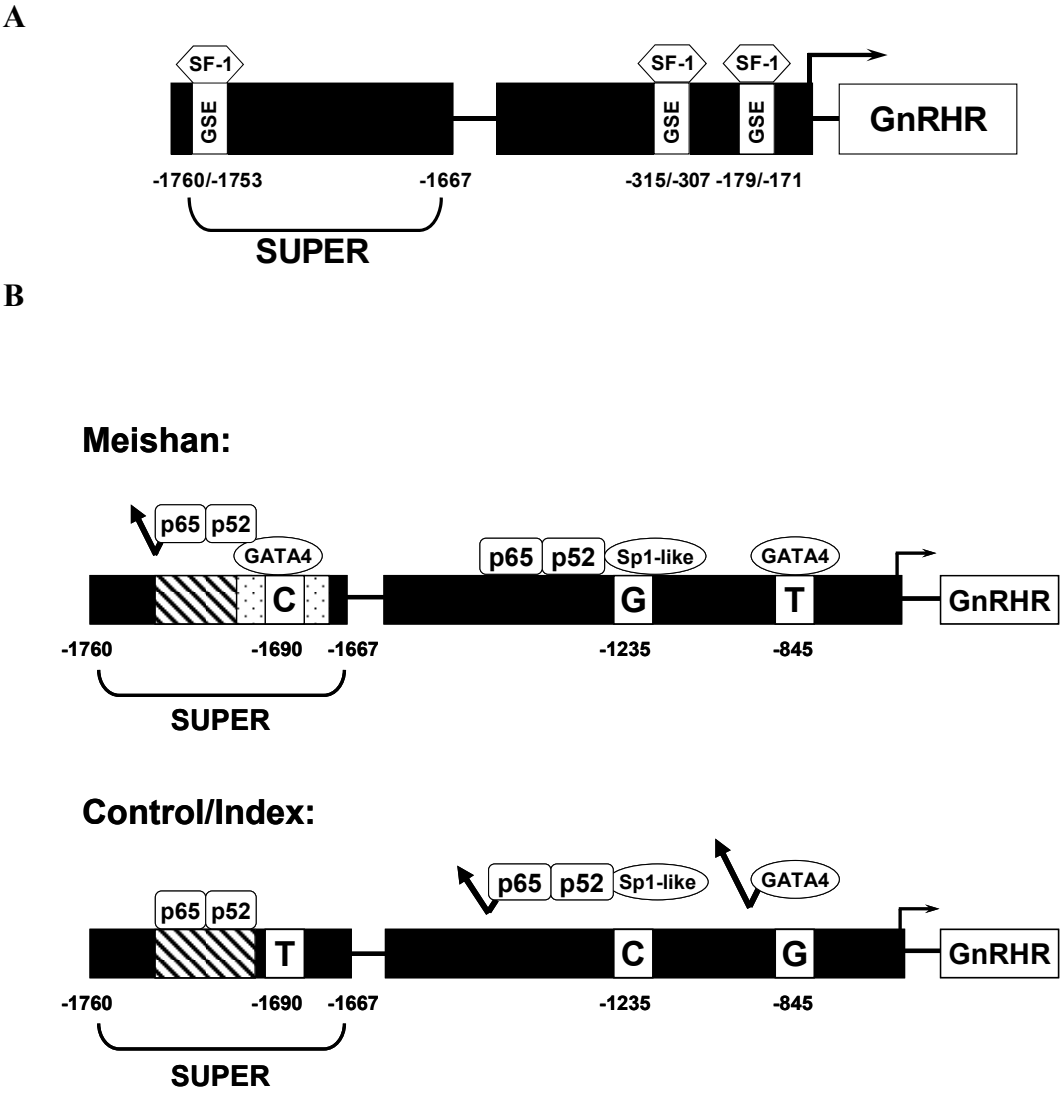


Figure 4.7. Working models for (A) cell- and (B) line-specific activity of the porcine GnRHR gene promoter. (A) In addition to the previously identified proximal SF-1 binding sites (-315/-307 and -179/-171), another SF-1 binding site (-1760/-1753) located within the important distal region of the promoter termed SUPER can be added to our working model for the cell-specific promoter of the porcine GnRHR gene. (B) Our laboratory previously identified a single bp alteration at -1235 bp of 5' flanking region allowing the p65/p52 subunits of NF-κB and an Sp1-like protein to bind to the Meishan GnRHR gene promoter. An additional bp substitution located at -845 bp of proximal promoter allows GATA-4 to bind to the Meishan promoter. None of these factors were capable of binding to the Control promoter. However, another polymorphism (-1690) within the critical distal portion of promoter called SUPER allows binding of the transcription factor GATA-4 to the Meishan promoter (GATA element, dotted box), whereas the p65 and p52 subunits of NF-kB bind to the homologous Control/Index promoters (NF-κB element, diagonal box).

Discussion

Our laboratory is primarily interested in transcriptional regulation of the porcine GnRHR gene. Recently, we identified a critical enhancer region of the porcine GnRHR gene promoter termed the swine upstream promoter enhancing region (SUPER) located at -1779/-1667 bp of 5' flanking region. Removal of this region resulted in a dramatic decrease in promoter activity (Cederberg et al., unpublished data). In this study, results from EMSA experiments further refined SUPER to -1760/-1667 bp of proximal promoter. In addition, we identified both cell- (-1760/-1753) and line-specific (-1690) elements within SUPER. Therefore, these results amplify the importance of SUPER in transcriptional regulation of the porcine GnRHR gene.

The first element identified within SUPER represents a new member of the cell-specific porcine GnRHR gene promoter. We identified an SF-1 binding site located at -1760/-1753 bp of proximal promoter. Previously, our laboratory identified 2 additional SF-1 elements located at -315/-307 and -179/-171 bp of proximal promoter that are also critical to cell-specific activity (Cederberg et al., unpublished data). Steroidogenic factor-1 binding sites are important in the regulation of the GnRHR gene in other mammalian species such as the mouse (Duval et al., 1997a), human (Ngan et al., 1999), sheep (Duval et al., 2000) and rat (Pincas et al., 2001). Additionally, SF-1 has also been implicated in the regulation of other gonadotrope-specific genes including the LHβ- (Keri and Nilson, 1996; Wolfe, 1999), FSHβ- (Brown and McNeilly, 1997; Jacobs et al., 2003) and α- (Barnhart and Mellon, 1994) subunit genes. Interestingly, the rat GnRHR receptor promoter is regulated by both distal and proximal regions, similar to the porcine GnRHR

gene promoter. In addition to a proximal tripartite enhancer originally identified in the mouse (Duval et al., 1997a), the rat GnRHR gene promoter is comprised of an important distal region, the GnRHR specific enhancer (GnSE; -1135/-753). Specifically, the GnSE helps confer gonadotrope-specific expression via its interaction with a proximal region located at -275/-226 bp of proximal promoter that includes an SF-1 binding site within the tripartite enhancer (Pincas et al., 2001).

The utilization of 3 SF-1 binding sites is unprecedented for gonadotropic genes. However, these recognition sites do not contribute equally to cell-specific activity of the porcine GnRHR gene promoter. Block replacement mutation of the 3' gonadotrope specific element (GSE) eliminated promoter activity, indicating that this SF-1 binding site is absolutely critical to expression of the GnRHR gene in gonadotrope derived αT3-1 cells. Mutation of the GSE located at -315/-307 bp reduced promoter activity by approximately 20% ($P < 0.05$; Cederberg et al., unpublished data). Consistent with this GSE, mutation of the SF-1 binding site located at -1760/-1753 bp identified in this study resulted in a 30% reduction in promoter activity. Thus, these 2 SF-1 recognition sites are necessary but not sufficient for expression of the porcine GnRHR gene in gonadotrope-derived cells. Transcription of some genes expressed within gonadotropes is regulated by 2 GSEs. The equine LHβ-subunit gene is regulated by 2 SF-1 sites located between -185/-100 and -55/-48 bp relative to the translational start site (Wolfe, 1999). Mutation of the 2 GSEs, individually (proximal or distal) or in combination decreased LHβ-subunit promoter activity by 14, 34, and 24% ($P < 0.05$), respectively in LβT2 cells. However, it did not attenuate basal expression of the promoter in αT3-1 cells (Wolfe, 1999). Thus,

the activity of the equine LHβ-subunit gene is partially conferred by 2 GSEs in LβT2 but not αT3-1 cells (Wolfe, 1999). The mouse FSHβ-subunit gene is regulated by 2 proximal GSEs and a downstream NF-Y element. Mutation of the 2 GSEs individually or in combination does not affect promoter activity in LβT2 cells. However, when the 2 GSEs were mutated in combination with the downstream NF-Y binding site promoter activity was reduced by 50% (Jacobs et al., 2003). The use of a single GSE by promoters for genes encoding the GnRHR is much more common. Functional activity of the mouse GnRHR gene promoter is partially conferred by an SF-1 binding site located at -244/-236 bp of 5' flanking region. Mutation of this GSE within the context of 600 bp of 5' flanking region reduced promoter activity by approximately 60% in αT3-1 cells (Duval et al., 1997a, 1997b). Activity of the human GnRHR gene promoter was increased by 150% upon overexpression of SF-1 in αT3-1 cells. Consistent with this, the addition of antisense oligonucleotides for SF-1 mRNA reduced activity of the human GnRHR gene promoter by 61% (Ngan et al., 1999).

The pig has served as a unique model to understand the relationship between the porcine GnRHR gene promoter and a QTL for ovulation rate located on chromosome 8. Three swine lines with divergent ovulation rates were utilized to analyze transcriptional regulation of the porcine GnRHR gene promoter; Control, Index and Chinese Meishan. Transient transfection of αT3-1 cells with reporter vectors containing the Control, Index and Meishan promoters indicated divergent activity among the lines of swine. Previously, our laboratory identified a single bp alteration unique to the Meishan located at -1235 of proximal promoter. This bp substitution allowed the p52 and p65 subunits of

NF-κB and an Sp1-like protein to bind to the Meishan promoter compared to the Control/Index promoters (McDonald, 2005). In the current study, we identified 2 additional single bp substitutions in the Meishan promoter, located at -845 and -1690 bp of proximal promoter. The single bp substitutions at both -845 and -1690 bp of 5' flanking region formed GATA-4 binding sites in the Meishan promoter. Block replacement mutations of the -845 and -1690 bp alterations within the context of the full-length Meishan promoter significantly reduced promoter activity by approximately 20 and 50%, respectively ($P < 0.05$), indicating that these elements contribute to the enhanced activity of the Meishan GnRHR gene promoter. In contrast to the Meishan-specific GATA-4 element, the single bp alteration within the Control/Index promoter located at -1690 bp of 5'flanking region formed an NF-κB binding site, interacting with the p65/p52 subunits of NF-κB. Block replacement mutations of this specific binding site within the context of the full length Control/Index promoter also significantly reduced promoter activity by approximately 60% ($P < 0.05$). Consequently, the 3 identified elements unique to the Meishan promoter contribute to its enhanced activity over the Control/Index promoters. Although it could be argued that the GATA-4 and NF-κB elements at -1690 contribute equally to activity of the Meishan and Control/Index promoter, respectively, since mutation of these sites reduced activity similarly (50% for Meishan vs. 60% for Control/Index promoters). However, it is still unknown how different elements within the porcine GnRHR gene promoter interact with one another.

To date, GATA-4 has not been implicated in the regulation of any GnRHR genes except the pig. However, GATA family members are important in the regulation of other

gonadotropic genes. For example, mutation of a GATA element identified in the human α-subunit gene promoter decreased activity by 2.5-fold in gonadotrope-derived αT3-1 cells and 15-fold in JEG-3 human placental cells, suggesting that GATA binding factors have a functional role in transcriptional regulation of the human α-subunit gene (Steger et al, 1994). Recognition sites for GATA factors have also been implicated in transcriptional regulation of genes within other reproductive tissues. GATA-4 is involved in transcription of several genes essential for gonadal development and function such as genes that encode the hormones, inhibin α and Mullerian inhibiting substance, the steroidogenic acute regulatory (sTAR) protein, and SF-1 (Tremblay and Viger, 2001). Binding sites for GATA proteins also play a role in activity of the neuronal-specific enhancer for the GnRH gene within the hypothalamus (Lawson et al., 1996). Further, gonadotropin-regulated genes in the adult ovary are controlled by GATA-4. GATA-4 mRNA and/or protein has been found in thecal and interstitial cells (McCoard et al., 2001; Gillio-Meina et al., 2003) as well as the granulosa cell layer of healthy follicles (Heikinheimo et al., 1997; McCoard et al., 2001; Vaskivuo et al., 2001; Gillio-Meina et al., 2003). In addition to the female, GATA-4 has also been detected in male reproductive tissues. Within the testis, GATA transcription factors have been identified in both Sertoli (Viger et al., 1998; Ketola et al., 1999; 2000; 2002; Anttonen et al., 2003;) and Leydig (Ketola et al., 1999; 2000; 2002) cells. Thus, GATA elements are abundant in genes associated with reproduction, playing an important role in transcriptional regulation of these genes.

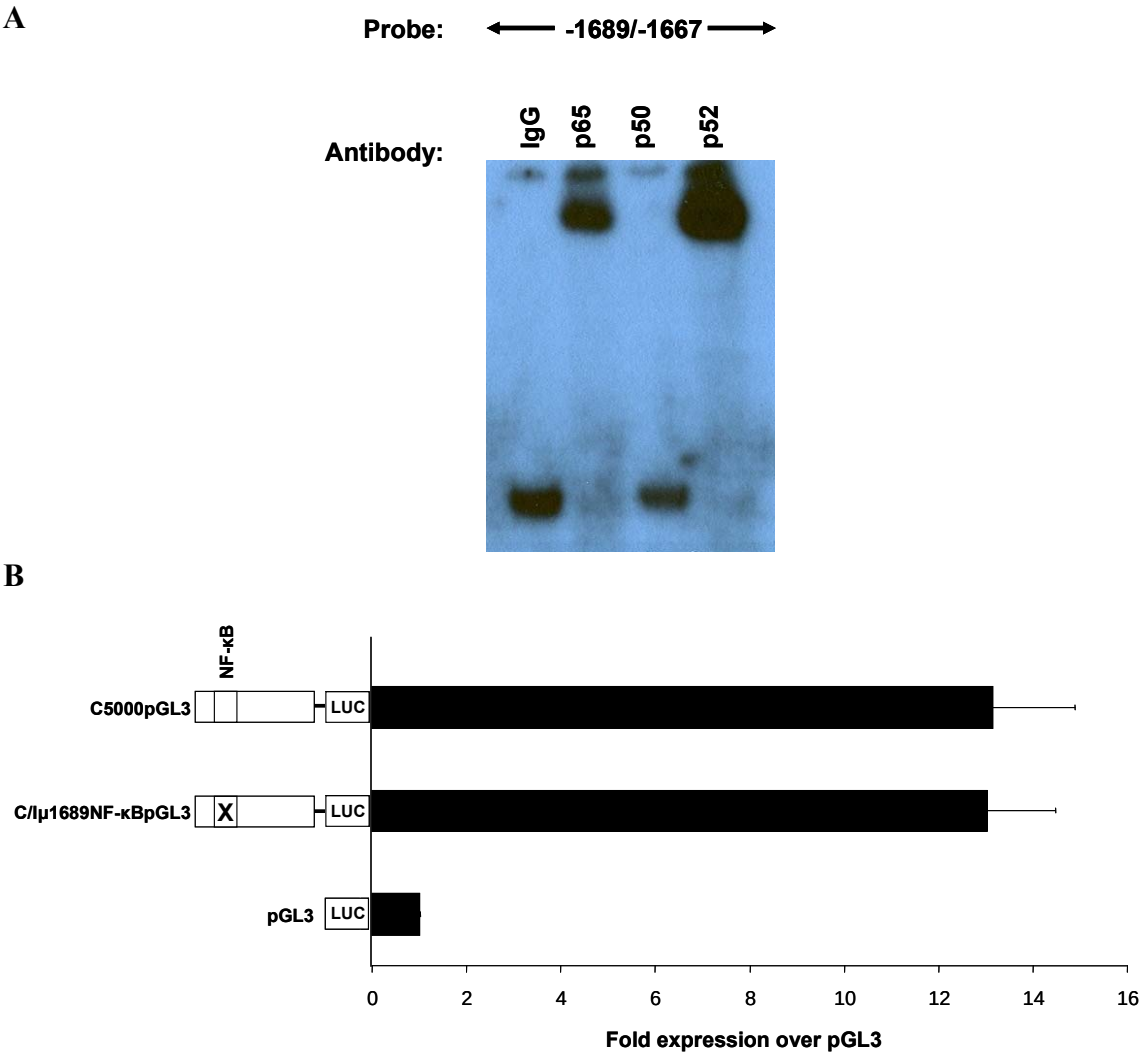
Previously, our laboratory identified a functional NF-κB element unique to the Meishan GnRHR gene promoter. In the current study, we also identified a functional NF-κB binding site specific to the homologous Control/Index GnRHR gene promoters located at -1690 bp of 5' flanking region. The transcription factor, NF-κB is important in regulating the immune system but also plays a role in embryonic development, the development and physiology of tissues (mammary gland, bone, and skin) and the central nervous system (Hayden and Ghosh, 2004). To date, NF-κB has not been linked to regulation of any other gonadotrope-specific genes besides the porcine GnRHR gene. Although NF-κB has not been implicated in the regulation of gonadotrope-specific genes, it can regulate other genes within the anterior pituitary such as the pituitary proopiomelancortin (POMC) gene. In response to stress, corticotropin-releasing hormone regulates NF-κB activity which is associated with activation of the pituitary POMC gene (Karalis et al., 2004). Although transcriptional regulation of downstream genes have not been investigated, activation of NF-κB complexes was detected in somatotrope cells following stimulation with interleukin-1 (Parnet et al., 2003). In addition to the important role NF-κB plays in transcriptional regulation of corticotropic and somatotropic genes within the anterior pituitary gland, the current study demonstrated that it is also critical in the regulation of gonadotropic genes (i.e., the porcine GnRHR gene).

Although the 3 elements unique to the Meishan promoter represented a portion of its enhanced activity in αT3-1 cells, further studies need to be performed to determine if additional binding sites contribute to its activity. For instance, reporter vectors

containing block replacement mutations of the 3 elements in combination should be utilized to determine if the enhanced GnRHR promoter activity in the Meishan line can be fully explained by only these 3 transcription factor binding sites. Despite the reduced luciferase activity in cells transfected with each Meishan block replacement mutation, however, these values still remained numerically higher than those obtained for the full-length Control promoter (15- to 25-fold over promoterless control). Thus, it is likely that other elements involved in transcriptional regulation of the Meishan GnRHR gene have yet to be identified.

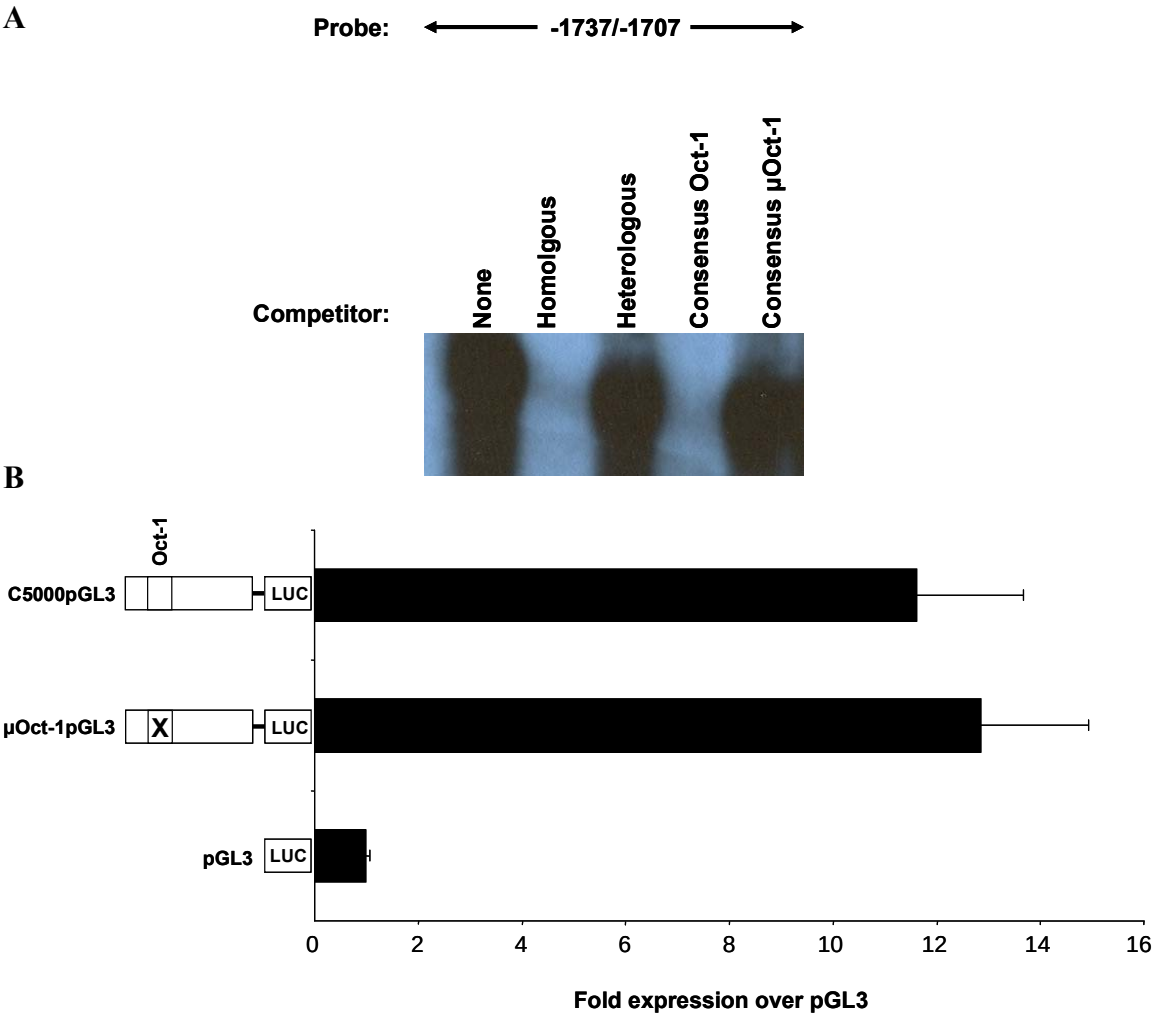
In summary, we added an additional SF-1 binding site located at -1760/-1753 bp of 5' flanking region to our working model for the cell-specific promoter of the porcine GnRHR gene (Figure 4.7A). Thus, we have demonstrated the importance of the 3 GSEs in transcriptional regulation of the porcine GnRHR gene. In addition to cell-specific elements, we identified 3 functional elements that contribute to the enhanced activity of the Meishan GnRHR gene promoter in αT3-1 cells (Figure 4.7B). Each respective element is formed due to single polymorphisms among lines within the GnRHR gene promoter. At -1235 bp of proximal promoter, the Meishan promoter contains a NF-κB and Sp1-like binding site, whereas 2 GATA-4 binding sites are located at -845 and -1690 bp of 5' flanking region. Alternatively, the single bp substitution at -1690 of proximal promoter maintains an NF-κB binding site within the Control/Index promoter. Ultimately, we could interchange the GnRHR gene promoter in Control and Meishan swine lines to determine if reciprocal GnRHR promoter exchange will result in a functional alteration of ovulation rate.

Appendix I



A) Electrophoretic mobility shift assays revealed a specific binding complex in the oligonucleotide spanning -1689/-1667 bp of the porcine GnRHR gene promoter. Addition of antibodies specific to the p65, p50 and p52 subunits of NF-κB or rabbit IgG resulted in a supershift of the p65 and p52 subunits of NF-κB. B) Reporter vectors containing the native, full length Control promoter (C5000pGL3), a block replacement mutation of the NF-κB element within the context of the full length Control promoter (C/Iμ1689NF-κBpGL3), or promoterless control (pGL3) were transiently transfected into αT3-1 cells. Luciferase (LUC) activity did not differ between the vector containing the block replacement mutation (C/Iμ1689NF-κBpGL3) and the full length promoter (C5000pGL3). Therefore, the identified NF-κB binding site did not contribute to functional activity of the porcine GnRHR gene promoter.

Appendix II



A) Examination of the oligonucleotide spanning -1737/-1707 bp of the porcine GnRHR gene promoter using EMSAs identified a specific binding complex. Addition of unlabeled oligonucleotides containing consensus binding sites for Oct-1 resulted in competition for binding. Consistent with this, addition of an unlabeled oligonucleotide containing a mutated Oct-1 (µOct-1) binding site was unable to ablate binding. This indicated the -1737/-1707 oligonucleotide contained an element that bound the Oct-1 transcription factor. B) Reporter vectors containing the native, full length Control promoter (C5000pGL3), a block replacement mutation of the Oct-1 element within the context of the full length Control promoter (µOct-1pGL3), or promoterless control (pGL3) were transiently transfected into αT3-1 cells. Luciferase (LUC) activity did not differ between the vector containing the block replacement mutation (µOct-1pGL3) and the full length promoter (C5000pGL3). Therefore, the identified Oct-1 binding site did not contribute to functional activity of the porcine GnRHR gene promoter.

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